ENHANCEMENT OF THE CYTOTOXIC ACTIVITY OF SOME α,β-UNSATURATED KETONES THROUGH AUXILIARY BINDING

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ABSTRACT

The antineoplastic properties of α,β-unsaturated ketones, such as curcumin and related analogs, have been investigated for a number of years. Due to the high light sensitivity and the low bioavailability of curcumin and its analogs, there was a need to modify its structural features for clinical usage as a chemotherapeutic candidate. The emphasis on synthetic curcumin analogs which have the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (Figure 2-1, page 32) and its importance for eliciting antineoplastic activity was made from this laboratory. It was proven that one way these agents exert their activity is through a thiol-alkylating mechanism. In order to increase the cytotoxic activity of these candidate agents, three target compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore were designed by incorporating potential auxiliary binder groups (Figure 2-2, page 33). The auxiliary binders may display antineoplastic properties, or they may act only to enhance the bonding at the binding site. The synthesis of the auxiliary binders and the target compounds containing different physicochemical properties was proposed in order to investigate the structural features responsible for enhancing the biological activity. Both auxiliary binders **1** and the target compounds **2** were synthesized successfully mainly through a series of condensation reactions (Schemes 4-1 and 4-2, pages 61-63). Following the synthesis, the biological evaluation was performed including multiple cytotoxicity assays[59-61] as well as a multidrug resistance (MDR)-revertant assay[62]. The compounds were analyzed via ^{1}H NMR, ^{13}C NMR, and CHN elemental analyses. The biological screening of **1a**, **1f**, and **1g** using four cell lines L1210, Molt4/C8, CEM, and HeLa cells revealed that they lack cytotoxic activity (IC_{50} > 100 μM, Table 4-1, page 65), and they might play a role in enhancing the

alignment of the target compounds to the receptor. However, other auxiliary binders also displayed very low cytotoxicity (IC_{50} > 100 μM) except for **1h** (IC_{50} = 61 μM). The target compounds **2c** (13.3 μM) and **2d** (15.3 μM) were more potent than **2b** (25.5 μM) in this assay.[59] These data are presented in Tables 4-1 and 4-2, pages 65 and 66. The target compounds also displayed anti-metastasis activity towards various non-adherent cancer cells[60] indicated on page 81. Generally, the target compounds **2b-d** have selectively inhibited tumor growth in the micromolar and sub-micromolar range, and the highest potency was displayed by **2b** which inhibited > 50% of non-adherent tumor cells at 1 μM. Two auxiliary binders inhibited more than 10% of the tumor cells at 5 μM namely, **1g** (20%, Jurkat) and **1j** (32.3%, DU145) respectively. These data are presented in Tables 4-4 and 4-5, pages 69 and 70. The selective cytotoxicity of the target compounds **2b-d** as well as the auxiliary binders **1** were also examined.[61] Some of the auxiliary binders displayed cytotoxic effects at CC_{50} < 80 μM namely, **1c**, **1f**, **1h**, **1j**, and **1m**. Compounds **2b-d** displayed potency in the range of CC_{50} = 14-44 μM; however, **2b** is the best candidate compound as it exhibited high selectivity (SI up to > 30.44) to cancer cells compared to **2b** and **2c**. Moreover the target compounds **2b-d** have MDR-revertant properties at low and high micromolar concentrations in which the established FAR values were > 1. Compound **2b** reversed the MDR at 50 μM (FAR = 118.15 at 50 μM) more that standard drug verapamil (FAR = 15.68). For future directions, selected auxiliary binders will be incorporated to the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore, and the mechanism of auxiliary binding should be investigated.

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I appreciate our office staff, Beryl, Jackie, Sandy, Claire and Angela for their help and colleagues namely, Swagatika, Muath, and Ahlam for their continued encouragement. Finally, I thank my family for their support and encouragement in this endeavor.

DEDICATION

This thesis is dedicated to my parents, Fawzeiah and Omar, whose constant support, prayers, and guidance have been my inspiration. I would like also to dedicate this thesis to my husband Bandar, whose unlimited support, love, and patience have yielded my success and last but not least, I would like to dedicate this thesis to my beloved kids, Fatimah, Ahmed, and Sumaia for their understanding and warm hugs.

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CC_{50}	the concentrations of the compounds required to kill 50% of the cells
DNA	Deoxyribonucleic acid
FAR	Fluorescence activity ratio
5-FU	5-fluorouracil
GSH	Glutathione
HER/neu	Human Epidermal Growth Factor Receptor 2
IC_{50}	the concentration of a compound required to inhibit the growth of the cells by 50%
MDR	Multidrug resistance
μM	Micromolar
p53	tumor protein 53
P-gp	permeability glycoprotein
RNA	Ribonucleic acid
SI	Selectivity Index
STAT3	signal transducer and activator of transcription 3

CHAPTER 1

1. Literature Review

1.1 Introduction

The term cancer was mentioned for the first time between 3000 BC and 1500 BC. It was written on Egyptian papyri and explained as cancer of the breast.[1] The word "cancer", which is derived from a Greek word meaning crab, was described in the 5th century BC as a group of diseases characterized by uncontrolled growth which could spread through the body in a life threatening way.[2,3] Cancer existed thousands of years ago, but the incidence rate was significantly lower in contrast to the current era. For the last fifty years, the probability of having cancer is twice as much as previously.[2] This situation refers to the increased acquisition of cancer-related habits such as smoking, unhealthy diets, and poor physical activity. Based on recent GLOBOCAN statistics, cancer is one of the leading causes of death globally, as it is the second and third leading causes in economically developed and developing countries, respectively. About 12.7 million new cases and 7.6 million deaths were estimated to occur in 2008 worldwide, and this number is expected to grow to 27 million new cases and 17.5 million deaths by 2050.[4,5] Despite cancer affecting people from different races, genders, social classes, and nations equally, the distribution of specific cancers among countries varies based on a number of factors. For example, the prevalence of smoking in economically developing countries leads to an increase in the incidence of lung cancer. Similarly, the increased demand on fast foods in European countries is a cause of the increased incidence of colorectal cancer.[6]

1.2 Cancer Biology

A detailed definition of cancer was given by Ruddon and colleagues as "an abnormal growth of cells caused by multiple changes in gene expression leading to a deregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host".[6] Deoxyribonucleic acid (DNA) is the genetic template of cells and is composed of genes. Malfunction of genes in different ways leads to abnormal changes in cell functions. These mutations could be gene deletion, amplification, translocation, or duplication.[6,7] Cancer initiation and development require 3-7 genetic hits for common cancers, or at least two genetic hits in some others.[8] These genetic mutations could be inherited before birth (spontaneous mutation), acquired after birth (somatic mutation), or both. Alternation of the expression of oncogenes and tumor suppressor genes results in interrupting the normal process of cell division and differentiation; hence this dysfunction produces new cells which prefer to grow abnormally while resisting death and are called tumors.[6,9] However, cancer development is not an easy process since the affected cells undergo mechanisms which can repair DNA genetic damages or induce apoptosis. Abnormal growing cells can be classified as benign or malignant tumors. Benign tumors invade surrounding tissues locally, while malignant tumors have the ability to invade neighboring tissues, spread through the lymphatic system, and metastasize to other remote organs in the body.[6] While each type of cancer possesses unique characteristics, even so all cancer types share the following features: decreased cellular differentiation, abnormal increased cellular proliferation, abnormal invasion ability, and the tendency to spread all through the body.

Cancers vary from each other in many ways because of different mutations. These factors are the growth rate, proliferation rate, cellular differentiation, onset age, invasion, metastasis, response to treatment, and prognosis. However, there are many mechanisms of gene mutation, either by DNA direct injury or by atypical genetic translation or transcription. The phenotypicity of cancer has clinical implications of how the disease is treated and what resistance to drug therapy may result.[2]

1.3 Treatment Options

A wide variety of treatment options are available for people who have been diagnosed with cancer based on the type and stage of the cancer. Both traditional and modern treatments are still applied, for example, but not limited to: radiation, chemotherapy, surgery, immunotherapy, targeted therapy, hypertherapy, and hormonal therapy. While the main goal of traditional cancer treatments is the complete removal of the tumor with minimal damage to surrounding normal tissues, a great deal of damage to normal cells generally occurs. However, novel cure therapies have shown significantly decreased damage to normal cells and tissues in contrast. Normally the treatment combines surgery followed by chemotherapy, radiation, or both as needed.

1.3.1 Surgery

Surgery is the oldest approach to cure cancer and the aim is to remove the tumor and terminate its spread to other organs. However, it has limitations since only non-metastasized solid tumors can be surgically removed. Many different techniques of surgery have developed recently such as laser surgery and electrosurgery. Laser surgery

is a technique which relies on focusing a laser light beam to cut through the tumor's tissue and is effective in reducing the cutting area as in traditional surgery. Laser surgery is applied only for certain cancers such as liver and rectal cancers. Electrosurgery is a technique involving the use of a high-frequency electrical current to destroy cancer cells. Cryosurgery techniques rely on using liquid nitrogen to freeze and kill the tumor. Many other techniques are also applied and used widely; overall, these techniques have significantly enhanced surgery efficacy and accuracy.[2]

1.3.2 Radiation

Radiation is a practical option to treat invaded or metastasized tumors, or even cancers which are difficult to be removed surgically such as in the brain. The type and magnitude of the radiation dose is based on the tumor location and stage. Ionized radiation such as high energy X rays attack DNA through free radicals which are generated by two different mechanisms. Reactive radicals activate the tumor protein 53 (p53) signaling pathway and induce apoptosis or block mitosis through damaging the chromosomes. Radiation has a small risk of causing cancer in the future, yet modern approaches have helped in reducing that risk, for example, by radiation planning and brachytherapy. In radiation planning, a 3D picture of the tumor is taken by X rays so that the radiation beam is directed specifically toward the tumor region resulting in reduced harm to the surrounding tissues. Brachytherapy involves the use of a radiation source (pellets) which emit very low doses of radiation on early stage cancers. Other techniques are applied using drugs that work to sensitize tumors followed by radiation therapy.

Hyperthermia is another technique which aims to sensitize tumor cells by increasing the temperature of the targeted tissue by a few degrees.[2]

1.3.3 Hormonal therapy

Some cancers are hormone-dependent that arise in particular tissues such as breast tissue. Various hormones, such as estrogens and androgens, stimulate the growth of these cancers, i.e., breast and prostate tumors, respectively. The main aim in this kind of treatment is to block the hormone receptors, namely estrogen positive (ER+) in breast and androgen receptor (AR) in prostate cancers, using inhibitors such as tamoxifen and flutamide. The inhibitors compete with the hormones at the target receptor and lead to a block of tumor growth. Another class of drugs act in reducing the enzyme required for the synthesis of the hormones, for example, estrogen and androgen synthases inhibitors. It should be noted that these inhibitors are less toxic in comparison to DNA-targeting anticancer agents, because they do not have an effect on DNA replication or division of the cells.[2,10]

1.3.4 Immunotherapy

Immunotherapy was proposed in the 1800s after the observation that tumors are able to regress in patients with bacterial infections, in which the immune system is stimulated to attack the bacterial invasion, and attacks cancer cells as well. This observation led to the development of protein molecules produced by the body itself, named the cytokines. Hence the main aim of immunotherapy is to prevent tumor metastasis and various therapies have been developed over the last decades such as

lymphokines, antibodies, and vaccines. Cancer immunotherapy could be classified into two main classes named active immunotherapy and passive immunotherapy. Active immunotherapy relies on the design of immunotherapeutic agents that induce apoptosis. On the other hand, passive immunotherapy involves the use of the patient's antibodies or other immune constituents to provide the body with the immunity to kill infections.[2,11,12]

1.3.5 Antiangiogenesis agents

Angiogenesis is the process in which new blood vessels grow from an established vasculature. It plays a significant role in normal cells as well as the growth and development of tumors. Developing tumors are not able to grow more than 2 mm in diameter without adequate supplies of nutrients and oxygen which is delivered through the angiogenesis process. Angiogenesis inhibitors are established as drugs and are now used in cancer treatment and other diseases. Bevacizumab (Avastin®) was the first antiangiogenesis drug developed and is used to treat many cancers such as colorectal, kidney, lung, and other neoplasms.[2,13]

1.3.6 Chemotherapy

Chemotherapy is a very widely used option and is applied to kill cancer cells by different mechanisms of action, basically through killing cancer cells or disrupting their proliferation. Chemotherapy is also applied to metastasized cancers individually or accompanying radiation, surgery, or both. Around 200 anticancer drugs are available today, and the choice of the drug is based on the cancer type and stage. Some of the classes of cancer chemotherapeutic drugs are: 1) alkylating and platinating agents 2)

antimetabolites 3) antibiotics 4) and plant-derived drugs. Alkylating agents, antimetabolites, and many others have distinct mechanisms of action, but in general share the feature of high reactivity to attack and damage DNA.[2,14] Herein are three general mechanisms of chemotherapeutic drugs.

1.3.6.1 Chemotherapeutic agents which damage the mitotic spindle

This class of chemotherapeutic agents acts by inhibiting the development of the mitotic spindle found in microtubules, a cytoskeletal protein which provides the structural support of chromosomes. Microtubules carry the chromosomes to dividing cells through a particular spindle movement and consequently leads to cell division. Mitotic inhibitors disrupt that spindle in a way that blocks cell division. Taxanes, Vinca alkaloids, and colchicine are common classes of mitosis inhibitors used to treat many cancers.[14]

1.3.6.2 Chemotherapeutic agents which block DNA synthesis

This class of drugs functions in blocking DNA synthesis and repair through disrupting essential DNA synthesis metabolic pathways. These molecules could either bind to essential enzymes required for chromosome replication and repair, and disrupt its function, or it could alter the DNA or ribonucleic acid (RNA) sequence i.e., cells become unable to replicate chromosomes and division is blocked. The molecules structurally resemble substances involved in normal cellular metabolism. For example, 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), and methotrexate are pyrimidine, purine, and folic acid, antimetabolites respectively.[14]

1.3.6.3 Chemotherapeutic agents which target DNA

Nitrogen mustard was the first alkylating agent to be used during World War I and it was noted that survived soldiers who had been attacked by the gas, developed a low number of white blood cells. Clinical determinations revealed that nitrogen mustard caused remission in a lymphoma. Hence, further attempts to discover and synthesize other alkylating agents followed. Nowadays, there are several alkylating agents used effectively to treat many cancers mostly in a combination with other treatments.[14]

This class of drugs acts by damaging DNA through crosslinking the DNA and disrupting its synthesis. They are highly reactive organic molecules and can cause mutagenicity and carcinogenicity. Alkylating agents attack various groups on the DNA molecules and modify the bases in DNA, which consequently results in affecting cellular functions in one of the following mechanisms based on the nature of the binding between the alkylating agents and the alkylated molecule:

1) Indirect DNA damaging, in which the attacked cells produce specific enzymes in order to repair alkylated DNA strands through cutting it out of the whole DNA. In the presence of a huge number of alkylated strands, chromosomes become fragmented and consequently malfunction.[10]

2) Non-permanent DNA crosslinking, in which the alkylating molecule binds to a DNA base preventing its normal pairing process. For example, alkylating the thymine

residues on DNA results in its pairing with guanine instead of adenine. Therefore, the DNA sequences become mutated, and the malfunctioning DNA sequences can be passed along to another generation of cells.[10]

3) Permanent DNA crosslinking, in which the alkylating molecules form a covalent crosslink between two strands of DNA. In the presence of many crosslinks, gene expression and chromosome, replication becomes blocked due to the inability of the strands to separate.[10]

Despite various problems accompanying cancer chemotherapy, it has shown very considerable therapeutic benefits in cancer treatment for decades. Selectivity, toxicity, and multidrug resistance (MDR) are related issues which lower the safety of anticancer drugs. The modern trend in anticancer drug design and development is focusing on designing molecules specifically targeting tumor cells based on the tumor profile with minimal damage to normal cells. As the complete removal of the cancer without damaging the rest of the body is the goal of treatment, target-based drug designing is a rational approach for developing novel anticancer agents with high selectivity to cancer cells compared to normal cells.

Glutathione **1** (GSH) (Figure 1-1) is a tripeptide has significant role in prevent cellular damage caused by reactive oxygen species. GSH contains sulfhydryl moiety which serves as a reducing agent for xenobiotics detoxification through formation of difulfide (GSSG) product. In cancer, GSH is a critical factor, that it regulates cellular

proliferation and apoptosis.[15] Elevated levels of GSH in some tumors were reported which protect cancer cells and consequently lead to cells survival.[16] Modulation of GSH levels in tumors is an approach in cancer chemotherapy.

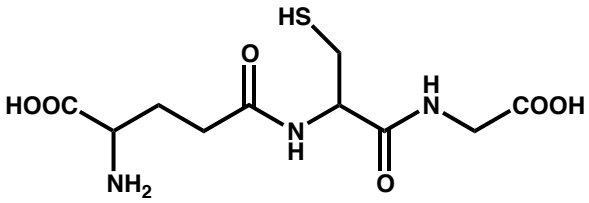


Figure 1-1: Glutathione structure **1**

In the present study, the aim is to enhance the cytotoxic potencies of some α,β-unsaturated ketones which are thiol-selective agents, and examine the structural features required to maximize the biological activity through auxiliary binding.

1.4 α,β-Unsaturated ketones

1.4.1 Introduction

Curcumin **2** (Figure 1-2) is a natural extract from the turmeric plant, the Indian spice used for a long time for cooking as well as for treatment purposes. In addition to its effect as an antineoplastic agent, curcumin also has anti-inflammatory, antioxidant, anti-diabetic, and antiangiogenic properties.[17] The biological activity of curcumin in inhibiting cancer cells proliferation through targeting various signaling pathways in cancer cells. However, due to the low oral bioavailability of curcumin, it has dimensioned its use clinically as a drug.[18] Many efforts in modifying the structure of curcumin were undertaken by medicinal chemists and yielded novel cytotoxins with enhanced

bioavailability. These structural modifications include the synthesis of dienones[19,20] which exhibit the cytotoxic potency through targeting GSH and deplete its levels in some tumors.[21]

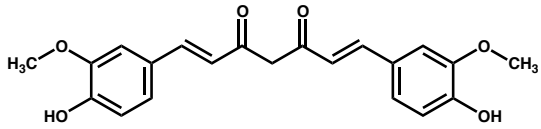
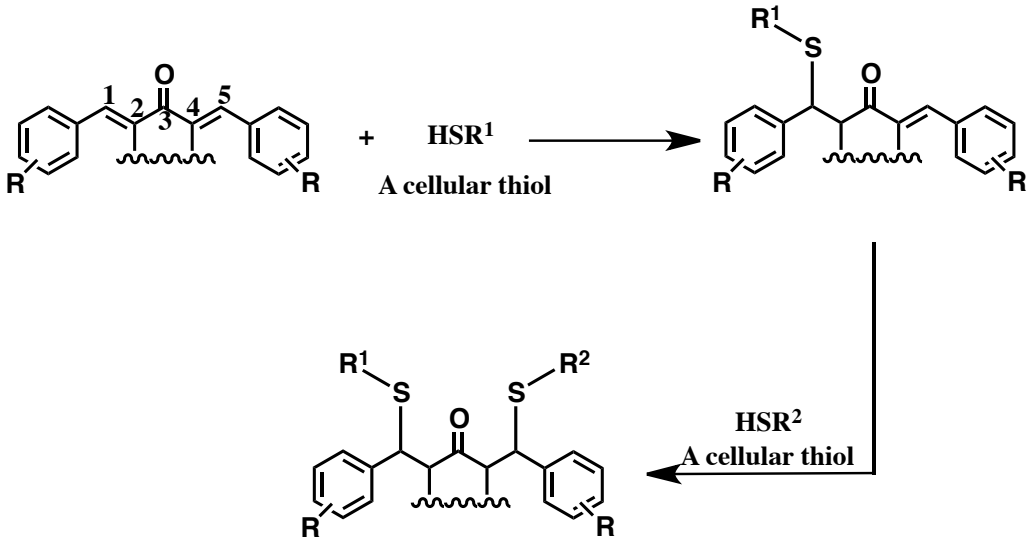


Figure 1-2: curcumin structure **2**

Acyclic conjugated α,β-unsaturated ketones, which resemble curcumin analogs, are thought to exert their effects through interactions with cellular thiols (Scheme 1-1). In addition, conjugated enones are thiol-selective agents[22], and as nucleic acids are thiol free, mutagenicity and carcinogenicity problems should be avoided in these agents. Chalcones, which are 1,3-diaryl-2-propenones, have confirmed the significance of conjugated α,β-unsaturated ketones in cancer chemotherapy. As a long-term project, this laboratory has worked on the development of a number of cyclic and acyclic conjugated unsaturated dienones which possess the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. Many of these compounds show potent cytotoxicity and anticancer properties. This series of compounds was developed in order to establish a correlation between structures and cytotoxic potencies. Substituted and unsubstituted aryl rings with different substituents were introduced which altered the polarity of the electrophilic centre as well as the hydrophobic and steric properties of the molecules.

Curcumin analogs have been extensively examined for the treatment and prevention of cancer and other diseases. In order to enhance the cytotoxicity of the molecules and the selectivity towards cancer cells rather than normal cells, a sequential cytotoxicity strategy was proposed. [23] Designing anticancer agents with multiple sites of action may minimize drug resistance and enhance selectivity.[24,25]



Scheme 1-1: The sequential alkylation of cellular thiols by compounds containg the 1,5-diaryl-3-oxo-1,4-pentadienyl group

1.4.2 Acyclic and cyclic α,β-unsaturated ketones

1.4.2.1 Acyclic α,β-unsaturated ketones

Many researchers have investigated various curcumin analogs in order to examine how these compounds exert their bioactivity. Lin et al. studied the effect of two synthetic acyclic curcumin analogs **3** (Figure 1-3) toward breast and prostate cancer cell lines. Both displayed greater cytotoxic potencies than curcumin itself and the IC_{50} values are

between 0.3-3.5 μM. As well, they inhibited AKT phosphorylation, downregulated HER/neu expression, and inhibited signal transducer and activator of transcription 3 (STAT3) signaling. Moreover, the compounds exhibited chemopreventive properties through the inhibition of cancer cells growth and migration.[26]

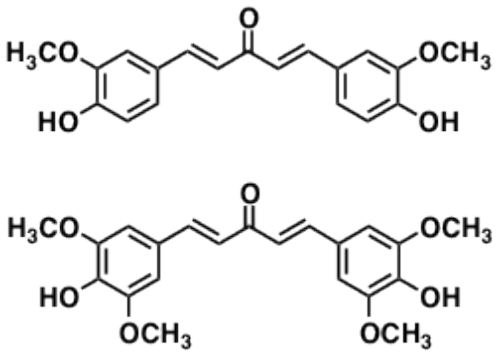


Figure 1-3: Acyclic curcumin analogs **3**

Appiah-Opong et al. have reported the inhibitory effect of various acyclic curcumin analogs on glutathione S-transferase (GST) in vitro and in vivo. The analogs **4** (Figure 1-4) displayed greater cytotoxic potencies than curcumin itself (IC_{50} values of 0.2-0.7 μM).[27]

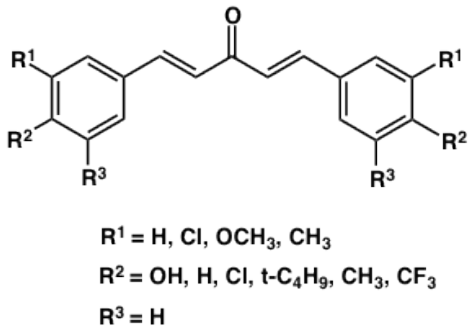
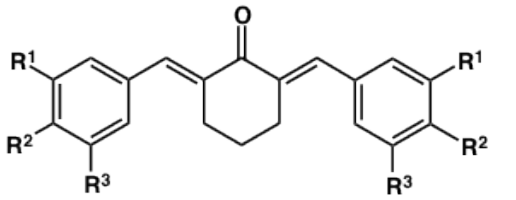


Figure 1-4: Acyclic curcumin analogs **4**

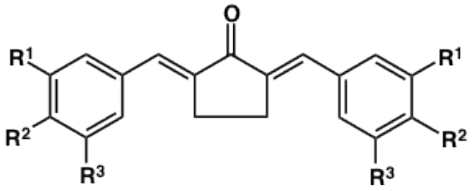
1.4.2.2 Cyclic α,β-unsaturated Ketones

Appiah-Opong et al. have also reported the inhibitory effect of various cyclic curcumin analogs on GST in vitro and in vivo. The analogs **5** and **6** (Figures 1-5 and 1-6) displayed greater cytotoxic potencies than curcumin itself (IC_{50} values of 0.2-0.7 μM).[27]



R^1 = H, Cl, CH_3, t-C_4H_9
R^2 = OH, H, OCH_3, CH_3, CF_3, $N(CH_3)_2$
R^3 = H, CH_3, t-C_4H_9

Figure 1-5: Cyclic curcumin analogs **5**



R^1 = H, Cl, CH_3, OCH_3, C_2H_5, i-C_3H_7, i-C_4H_9
R^2 = OH, H, Cl, OCH_3, CF_3, $N(CH_3)_2$
R^3 = H, CH_3, i-C_4H_9, i-C_3H_7, C_2H_5

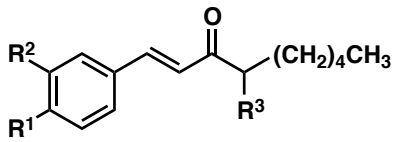
Figure 1-6: Cyclic curcumin analogs **6**

1.4.3 Mannich bases

1.4.3.1 Acyclic Mannich bases

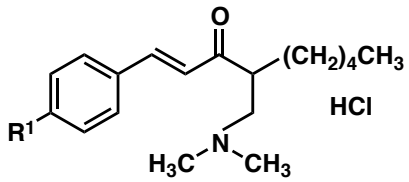
A series of acyclic Mannich bases, which are β-aminoketones, was designed and evaluated against cancer cells. The basic compounds in series **7** and **8** (Figures 1-7 and 1-

8) have shown anticancer and cytotoxic properties.[28] In addition, they have shown high reaction rates towards a thiol.[29] However, unwanted toxicity levels in mice were reported.[28,30] This may be due to the cyclic enones' flexibility.



$R^1 = R^2 = H, Cl$
$R^3 = CH_2N(CH_3)_2HCl$

Figure 1-7: Acyclic Mannich bases **7**



$R^1 = H, Cl$

Figure 1-8: Acyclic Mannich bases **8**

1.4.3.2 Cyclic Mannich bases

1.4.3.2.1 3,5-bis(Benzylidene)-4-piperidones

The drawback of acyclic Mannich bases led to the decision of designing cyclic Mannich bases which are more rigid molecules. The arylidene keto group and amine centre of previous molecules was retained. The flexibility was reduced by incorporating a piperidine ring into the structure. Additionally another arylidene group was introduced with a view to increase the cytotoxic potency because of the second alkylating group in **9**

(Figure 1-9). The resultant enhanced cytotoxicity motivated further modifications[25] including various quaternary and non-quaternary salts **10** (Figure 1-10) which showed similar cytotoxic potencies. The quaternary ammonium salts may have mutagenic potential as they bind to the DNA minor groove.[31-33]

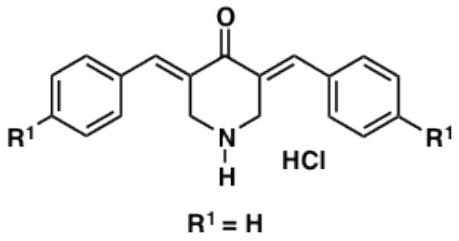


Figure 1-9: Cyclic Mannich base **9**

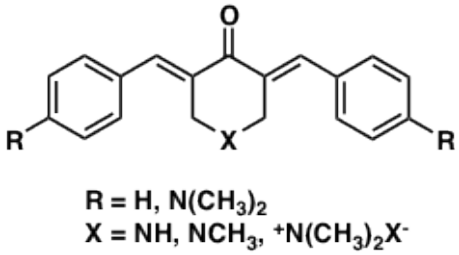


Figure 1-10: Cyclic Mannich bases **10**

Sun el al. prepared the prodrugs of some curcumin analogs **11** (Figure 1-11). The analogs were treated with glutathione (GSH) and cysteine-containing dipeptides to form conjugate analogs. The antitumor activity was retained, but the conjugate prodrugs have enhanced water solubility and light stability compared to the parent analogs **12** and **13** (Figure 1-12).[34]

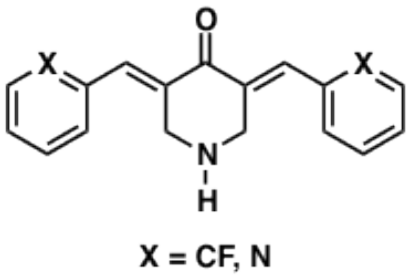


Figure 1-11: Cyclic Mannich bases **11**

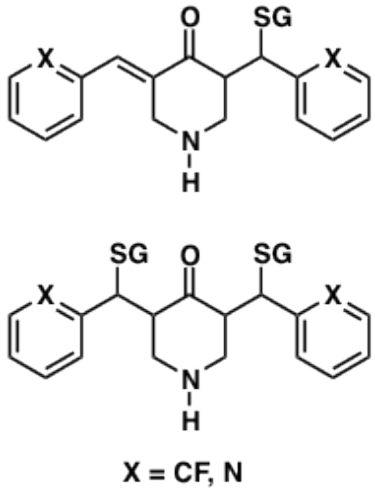
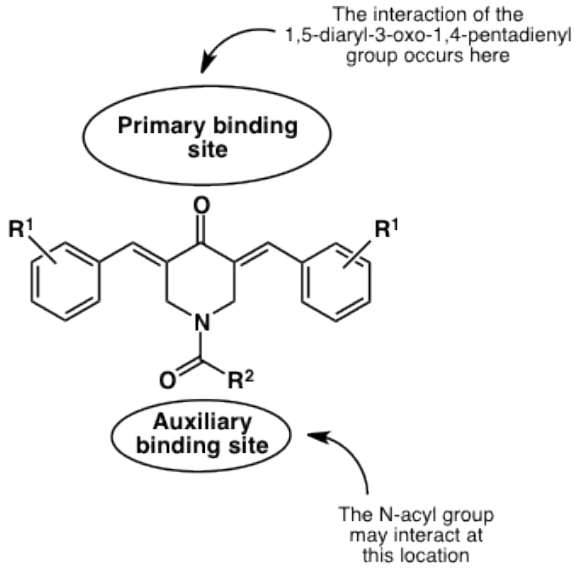


Figure 1-12: Adducts **12** and **13**

1.4.3.2.2 N-Acyl-3,5-bis(benzylidene)-4-piperidones

Further molecular modifications were conducted on the compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group. An acyl group was introduced onto the piperidyl nitrogen atom based on the assumption that it might increase the potency of the molecules through auxiliary binding with a receptor. Also it might enhance membrane permeability to malignant cells by functioning as a prodrug (Scheme 1-2).[35]



Scheme 1-2: Proposed primary and auxiliary binding sites

The N-Acyl groups were introduced first to the 3,5-bis(benzylidene)-4-piperidone **14** (Figure 1-13) by incorporating an additional thiol alkylating site **15** (Figure 1-14) or forming a prodrug **16** (Figure 1-15). Some of the compounds in series **15** and **16** were screened ($R^1 = R^2 = H$) and have shown increased potencies in comparison to the non N-acyl compounds; moreover, some of the compounds in series **15** and **16** ($R^1 = R^2 = Cl$) have exhibited very potent cytotoxicity with lower IC_{50} values than the figure for melphalan.[32,36]

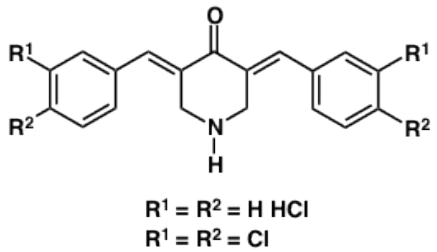


Figure 1-13: Cyclic Mannich bases **14**

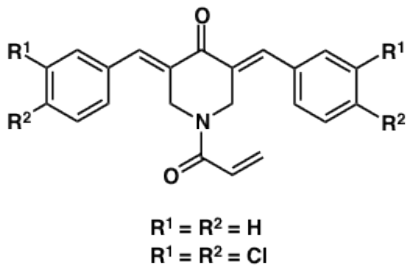


Figure 1-14: Cyclic Mannich bases **15**

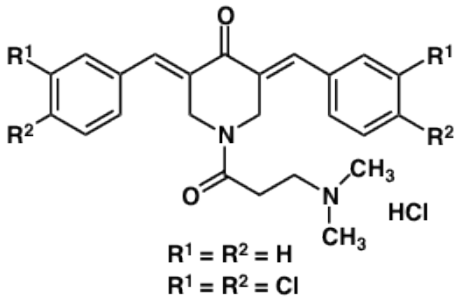


Figure 1-15: Cyclic Mannich bases **16**

The importance of the 1,5-diaryl-3-oxo-1,4-pentadienyl group was shown by the replacement of the keto group in the free base of the Mannich base **9** (Figure 1-9) by an alcohol function **17** (Figure 1-14) which resulted in an increased IC_{50} value.[36] In addition, the liberation of the N-acyl group of some N-acyl analogs **15** and **16** ($R^1 = R^2 = H$) (Figure 1-12 and 1-13) was confirmed along with other metabolites.

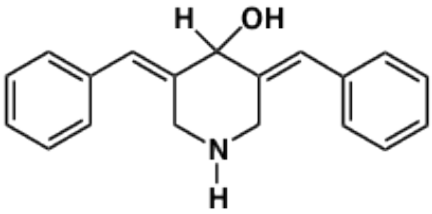
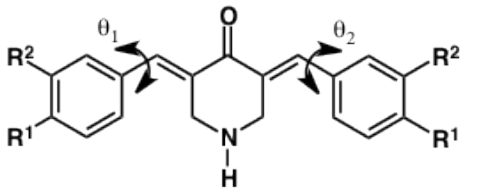


Figure 1-16: Replacement of keto group with hydroxy group in **17**

More investigations were made in order to examine the relative cytotoxic potencies of series **14** and **15** (Figures 1-13) and (Figures 1-14) in the presence of different groups on the ring with different electronic properties. The series of compounds **18** (Figure 1-17) and **19** (Figure 1-18) were screened against different cancer cell lines and series **19** possesses increased cytotoxic potencies compared to series **18**, which may be due to the additional alkylating site.[35] It was proposed that the cytotoxic potencies of the molecules are influenced by the torsion angles θ between the olefinic groups and the aryl rings. In this case potency was increased as the θ values were elevated. The importance of the θ values in influencing potencies in bioevaluations has been articulated elsewhere.[37,38] In particular, both **18e** and **19e** inhibited RNA and protein synthesis and induced apoptosis.[35]



a $R^1 = R^2 = H$ d $R^1 = F$; $R^2 = H$
b $R^1 = Cl$; $R^2 = H$ e $R^1 = NO_2$; $R^2 = H$
c $R^1 = R^2 = Cl$ f $R^1 = OCH_3$; $R^2 = H$

Figure 1-17: Cyclic Mannich bases **18**

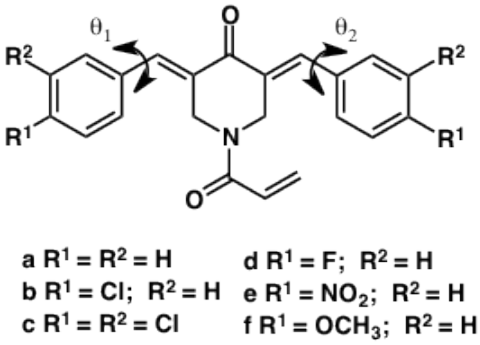


Figure 1-18: Cyclic Mannich bases **19**

In order to confirm the importance of the N-acyl groups in enhancing the auxiliary binding at a receptor, the analogs **9** (Fig 1-9) were synthesized with different bulky substituents and different basic centers **20** (Figure 1-19) and **21** (Figure 1-20). In general, they show increased in vitro potency than the parent compound **9** (Figure 1-9) (IC_{50} 0.52-11.5 μM). In particular, series **21** are more potent than compounds in series **20** which may be due to increased bonding to an auxiliary binding site.[39] In addition, they reverse P-gp associated multidrug resistance.[40] Some compounds in series **21** (R^1=Cl; R^2= 4-morpholinyl) exhibited very high toxicity towards colon cancer HCC-2998 with an IC_{50} figure of less than 5 nM.[39]

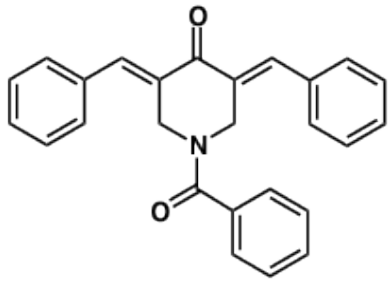


Figure 1-19: Cyclic Mannich base **20**

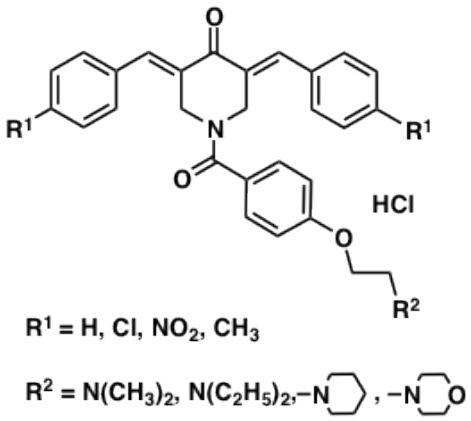


Figure 1-20: Cyclic Mannich bases **21**

1.4.3.2.3 Other N-acyl analogs of 3,5-bis(benzylidene)-4-piperidones

An additional study was conducted in order to examine the tolerance at the binding sites. Various series of compounds were designed in which the chalcone and cinnamoyl motifs were introduced as a N-acyl group **22a** and **22b**, respectively (Figure 1-21).[41] The rationale was to provide an additional alkylating site, to maximize binding to a receptor and to enhance the targeting of the molecules. However in general compounds **22a** and **22b** were less potent in vitro than the lead compound **9** (Figure 1-9). Subsequent modifications led to the synthesis of compounds **22c** and **22d**.[42,43] Many of these compounds were more potent than melphalan towards Molt4/C8 and CEM T-lymphocytes and exhibited potent cytotoxicity of IC_{50} values less than 5 μM. From the point of view that human N-myristoyltransferase (hNMT) enzyme is believed to be involved in the myristoylation process[44] and its activity in some colorectal cancers is greater than in the corresponding normal tissues, selected compounds in series **21** and **22** were screened in order to examine their effect on hNMT. However no inhibition of

hNMT was observed at concentrations which inhibited the growth of the neoplastic and transformed cells.

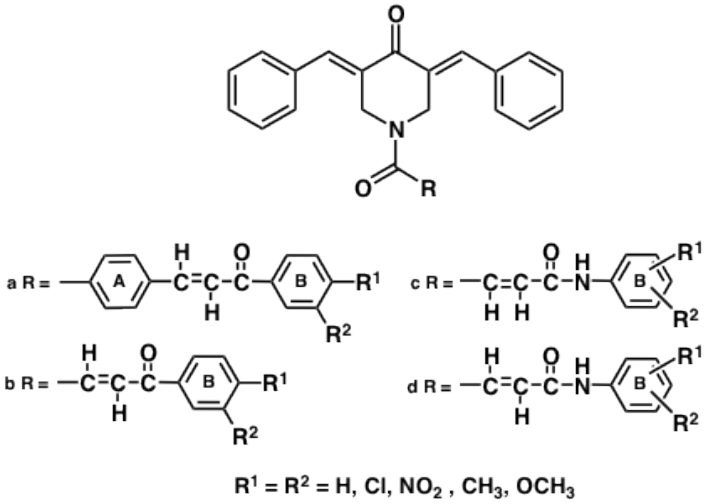
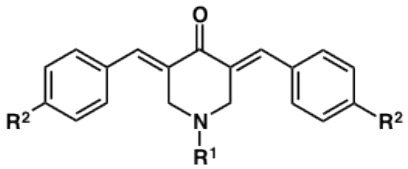


Figure 1-21: Cyclic Mannich bases **22**

Further studies were conducted in order to examine and increase the potential of the lead compounds in series **18** (Figure 1-17) and **19** (Figure 1-18) and to create molecules which demonstrated selective cytotoxicity toward malignant rather than normal cells. Based on the observation that co-administration of sodium 2-sulfanylethanesulfonate (mesna) and anticancer drugs may cause reduced side effects to normal cells, a series of adducts **23** were prepared (Figure 1-22).[45] In general the compounds in series **23** when R^1 is hydrogen or the N-acyl group have lower CC_{50} values to HSC-2, HSC-4, and HL-60 tumor cell lines than melphalan. These compounds were less toxic towards HGF, HPC, and HPLF non-malignant cells and hence demonstrated selectivity to the neoplasms. On the other hand, the mesna adducts (R^1= $COCH_2CH_2SCH_2CH_2SO_2OH$) had higher CC_{50} values and less tumor-selectivity.



R^2 = H, Cl, NO_2, CH_3, OCH_3

R^1 = H, $COCH{=}CH_2$, $COCH_2CH_2SCH_2CH_2SO_3H$

Figure 1-22: Adduct analogs **23**

Examining the influence of the cyclic scaffold on cytotoxic potency was also reported. Compound **24** (Figure 1-23), which is an acyclic analog of the cyclic compound **23** (R^1= H; R^2= NO_2) was prepared and displayed 20 times reduced potency in comparison to compound **23** (R^1= H; R^2=NO_2). It was concluded that the retention of the piperidine ring is very important in terms of cytotoxicity.[45]

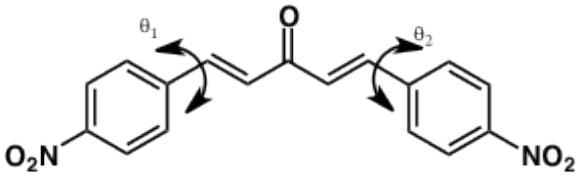
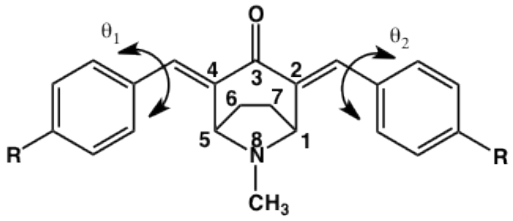


Figure 1-23: Compound's **23** acyclic analog **24**

Also, the torsion angles θ_1 and θ_2 were studied and in series **23** were significantly higher than the corresponding nitro analog **24**, which may contribute to the reduced cytotoxicity of the acyclic analog.

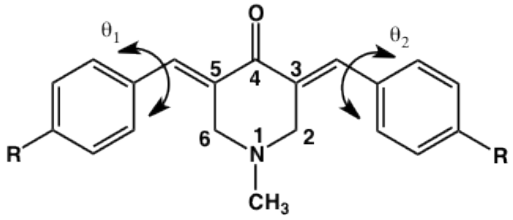
The importance of the nature of the cyclic scaffold was shown through series **25** (Figure 1-24) which have a dimethylene bridge attached to carbons 1 and 5 of piperidine

ring. Biological screening of series **25** and bridge-free analogs **26** (Figure 1-25) revealed that the presence of the dimethylene bridge led to compounds with reduced cytotoxic potency.[46]



R = H, Cl, NO_2, CH_3, OCH_3

Figure 1-24: Bridge-attached analog **25**



R = H, Cl, NO_2, CH_3, OCH_3

Figure 1-25: Bridge-free analog **26**

In addition to the benefits of using curcumin analogs as antitumor and antineoplastic agents, the effect of applying them as adjuvant chemotherapy agents has been studied. Kanwar et al. studied the effect of curcumin analog **27** (Figure 1-26) as an inhibitor of colon cancer stem-like cells which have a role in cancer recurrence. Curcumin analog 20 was used in combination with both 5-FU and oxaliplatin (Ox) for colon cancer treatment, and it showed an effective approach to prevent colon cancer recurrence through colon cancer stem-like cells elimination.[47]

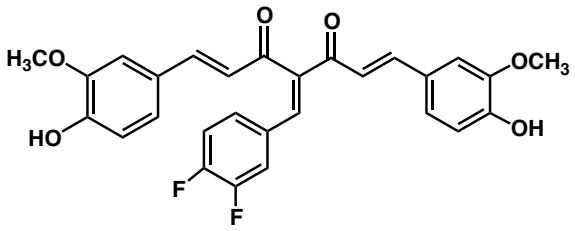


Figure 1-26: Curcumin analog **27**

Faiao-Flores et al. also reported the effect of using curcumin analogs as adjuvant chemotherapeutic agents with paclitaxel for breast cancer treatment in vivo. It was concluded that the combination therapy increased apoptosis up to 40% in tumor cells through caspase-3 phosphorylation activation.[48] Abuzeid et al. investigated the effect of a curcumin analog **28** namely FLLL32 (Figure 1-27), on the antitumor activity of cisplatin. Pre-administration of FLLL32 induced the antitumor effect and sensitized cancer cells to cisplatin. The compound exhibited its bioactivity through inhibiting STAT3 phosphorylation, which consequently induced apoptosis and sensitized cancer cells to chemotherapy treatment.[49]

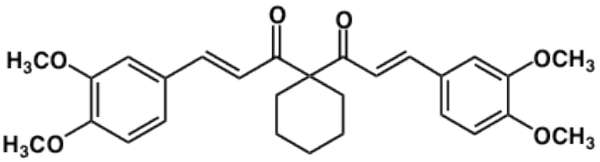


Figure 1-27: The FLLL32 structure **28**

1.4.4 Multidrug Resistance

1.4.4.1 Introduction

Multidrug resistance (MDR) development is a major problem in cancer chemotherapy and there is an urgent need for developing drugs which are capable of reversing MDR. The drug resistance takes place in tumor cells when the drug efflux increases and the intracellular concentration of the drug is diminished leading to a reduction of the treatment effectiveness and thereby giving rise to undesirable outcomes. P-gp is a member of the ABC transporters located across the cellular membrane, and is encoded by mdr-1 and mdr-3 genes. It functions in transporting a wide variety of substrates such as ions, amino acids, proteins, and xenobiotics. The phenomenon of MDR might result from genetic mutations induced by the antitumor agents used in the treatment, or it might be due to a pre-existing resistance in the tumor to the drug. MDR-revertants exhibit their properties by 1) binding to P-gp and thus inhibit the drug efflux, and 2) reducing the P-gp activity or its overexpression.[50,51]

1.4.4.2 MDR-revertant properties of α,β-unsaturated ketones

A number of naturally occurring curcumins and synthetic analogs, in addition to curcumin itself, have demonstrated their abilites to overcome drug resistance, increase drug accumulation in the cells, and decrease the drug efflux by various mechanisms. These compounds have a similarity to the curcumin pharmacophore, namely an α,β-unsaturated group which is likely responsible for the MDR-revertant activity.

Limtrakul and colleagues have reported that pure curcumin **2** (Figure 1-2) and the naturally occurring analogs, demethoxycurcumin **29** and bisdemethoxycurcumin **30** (Figure 1-28) have reversed human MDR activity. All three compounds have decreased the expression of the mdr-1 gene in KB-V1 cervical cancer cells, most significantly by bisdemethoxycurcumin which displayed the maximum revertant activity (Figure 1-26).[52]

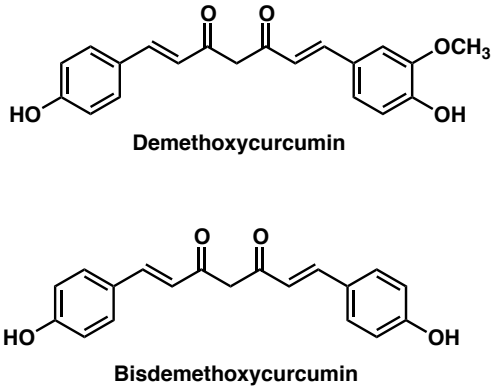


Figure 1-28: Structures of demethoxycurcumin **29** and bisdemethoxycurcumin **30**

Further investigation was also made to examine the effect of tetrahydrocurcumin **31** (THC) (Figure 1-29) on three P-gp proteins; ABCB1, ABCG2, and ABCC1 using various human cervical carcinoma and breast cancer cells. It was found that THC inhibited the three P-gps hyperactivity in a dose-dependent manner by binding to the ABC transporter, and the accumulation of the fluorescent dye was confirmed in the cells.[53]

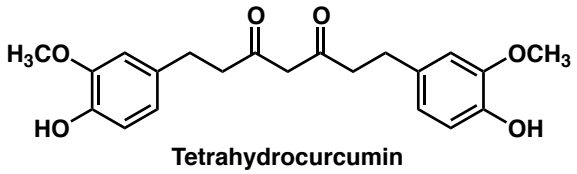


Figure 1-29: Structure of tetrahydrocurcumin **31**

Another study by Anuchapreeda and colleagues was carried out to investigate the effect of curcumin on MDR-resistance activity on human cervical carcinoma KB-V1 cells in vitro. The study reported that pure curcumin modulated the expression and activity of MDR P-gp in the cells using the range of concentrations of 1-55 μM.[54] Choi and colleagues carried out a study in order to investigate the mechanism of MDR-revertants in the L1210 cell line. They found that mdr-1b inhibition was made through the inhibition of the PI3K/Akt/NF-jB signaling pathway by curcumin.[55] More complex analogs of curcumin which exhibited MDR-revertant activity have been reported. Das and colleagues described a number of α,β-unsaturated ketones which possesses potent P-gp MDR revertant activity at low micrograms per mL in murine L5178 lymphoma and Colo 320 colonic carcinoma cells. FAR values of higher than 1 indicate that the MDR-reversal has taken place. For example, compound **32** (Figure 1-30) reverted MDR in both L5178Y/MDR-1 and Colo 320/MDR-1 cells at concentrations of 0.4 and 4 μg/mL.[40]

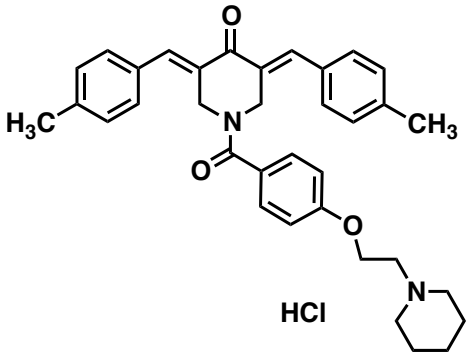
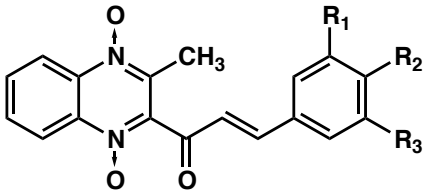


Figure 1-30: MDR-revertant **32**

Other α,β-unsaturated ketones were also reported from this laboratory as MDR-revertants. Series **33** (Figure 1-31) in general have FAR values of ≥ 1 in both L5178Y/MDR-1 and Colo 320/MDR-1 assays, to some extent, in a dose-dependent manner. Based on these observations, it was suggested that the 3-aryl-2-propenoyl group is significant for the MDR-revertant activity in these compounds.[56]



	R_1	R_2	R_3		R_1	R_2	R_3
a	H	H	H	**g**	H	Cl	H
b	H	OCH_3	H	**h**	Cl	Cl	H
c	OCH_3	OCH_3	H	**i**	H	Br	H
d	OCH_3	OCH_3	OCH_3	**j**	H	F	H
e	OCH_2O		H	**k**	H	NO_2	H
f	H	CH_3	H	**l**	NO_2	H	H

Figure 1-31: MDR-revertant **33**

1.5 Conclusion

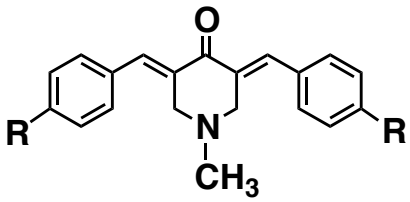
Chemotherapy is one of the widely used approaches in cancer treatment. It is aggressive toward tumors and has proven its efficacy in curing cancer for decades. The success of the treatment depends on many factors such as the patient's health and the type of cancer. However the treatment side effects can outweigh the benefits. Hence, cancer researchers are working on the development of anticancer agents which can kill tumors without damaging healthy cells. Curcumin analogs are a class of anticancer agents which have multiple biological effects such as anticancer and antioxidant properties in addition to their inhibitory effect on some enzymes which are involved in cancer initiation and development. The synthetic analogs have shown encouraging anticancer and MDR-revertant properties in cancer chemotherapy. Based on the physicochemical properties, the biological activity of these agents varies. A number of curcumin analogs are candidate anticancer drugs and further research is required in order to fulfill the demand for finding better chemotherapeutic agents.

CHAPTER 2

2. Purpose of the project

2.1 Rationale

The preparation of series **1** (Figure 2-1) as candidate antineoplastic agents was reported from this laboratory few years ago.[46] The bioevaluations towards human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 lymphocytic leukemia cells was undertaken. When discussing the relative potencies in this chapter of the thesis, the average of the IC_{50} values of the compounds in these three screens are presented. Series **1** was designed based on the hypothesis that the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore interacts at a primary binding site which is responsible for the bioactivity. The unsubstituted compound **1a** has an IC_{50} value of 4.69 μM in comparison to a reference drug melphalan which has an average IC_{50} figure of 2.61 μM. Obviously the unsubstituted analog is a lead compound.



1a R= H

1b R= CH_3

1c R= OCH_3

Figure 2-1: Structure of series **1**

Hence, the decision was made to investigate whether placing various substituents in the aryl rings could lead to analogs with increased potencies. In particular, the aim was to prepare compounds that could align at an auxiliary binding site in addition to interaction of the 1,5-diaryl-3-oxo-1,4-pentadienyl group at a primary binding site. This idea is illustrated in Figure 2-2. Small groups capable of binding through hydrophobic **1b** or hydrophobic and hydrogen bonding **1c** (Figure 2-2) were placed into the aryl rings which led to compounds having average IC_{50} values of 171 and 228 μM, respectively.[46] Therefore, the decision was made to place substantially more complex groups onto the aryl rings which may interact at auxiliary binding sites. These aryl substituents (designated R in Figure 2-2) are referred to subsequently as auxiliary binders. These auxiliary binders should contain one or more atoms or groups capable of establishing ionic, hydrogen, and van der Waals bonding with complementary atoms or groups in neoplastic cells.

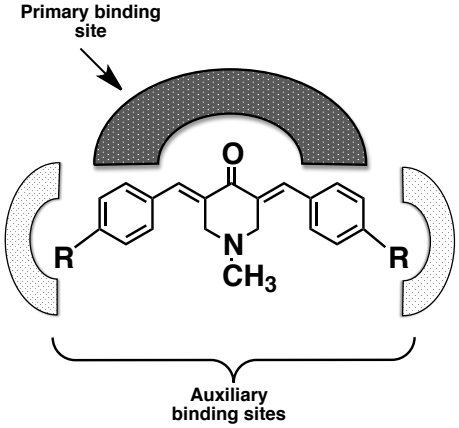


Figure 2-2: Proposed primary and auxiliary binding sites

Previously a project was undertaken with compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore which had an additional group or the piperidyl nitrogen atom which permitted supplementary bonding. Thus in series **2** (Figure 2-3), the compound can react at the primary binding site while in the analogs **3** (Figure 2-3), additional bonding by the group attached to the piperidyl nitrogen atom could take place at an auxiliary binding site.[39] A contrast between the cytotoxic potencies of series **2** and **3** in which the aryl substituents are identical revealed that in 48% of the comparisons, greater potency was demonstrated in series **3** while in 35% of the cases, equipotency was observed. Hence, the long-term decision was made to synthesize and evaluate compounds with the general formula **4** and related compounds (Figure 2-3).

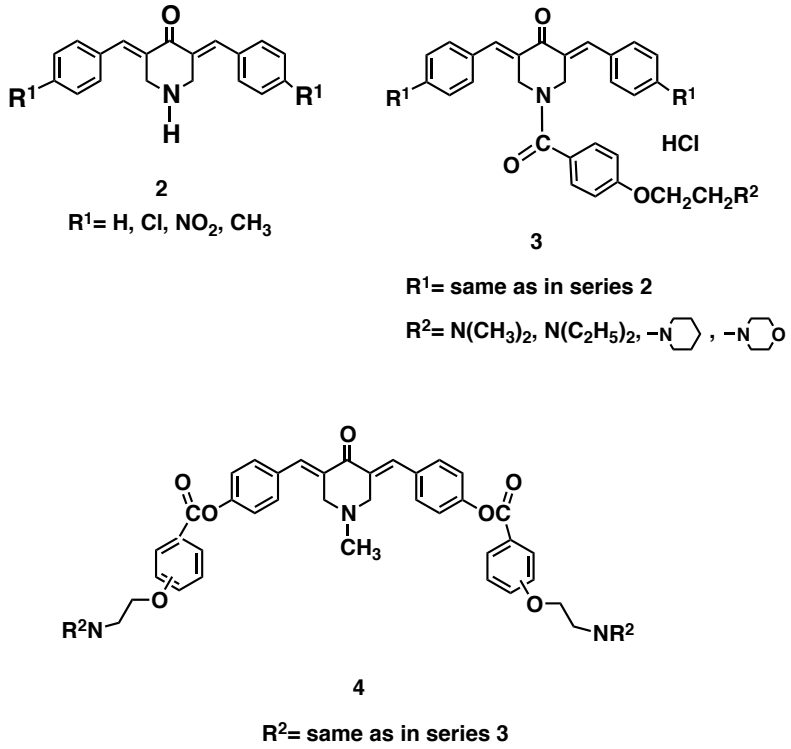


Figure 2-3: Structures of series **2**, **3**, and **4**

2.2 Hypothesis

The purpose of the present investigation is to evaluate the following suppositions:

1) A series of candidate auxiliary binders will have low or undetectable cytotoxic properties per se (if the auxiliary binders display antineoplastic properties, they would be acting at sites far removed from the primary binding site).
2) The potencies of cytotoxins containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore will be increased when linked to the auxiliary binders.
3) The target compounds will demonstrate tumor-selectivity.

CHAPTER 3

3. Syntheses and bioevaluations

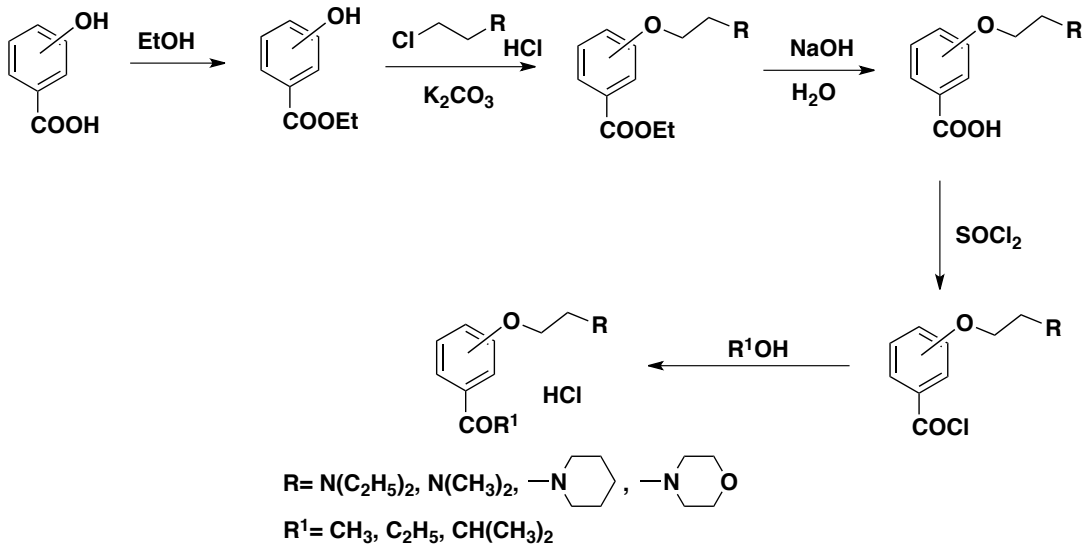
3.1 Syntheses

3.1.1 Materials and Methods

3.1.1.1 Synthesis of Series **1**

3.1.1.1.17 General scheme for the syntheses of the compounds **1a-c**,**e-g**,**j-l**.

A general methodology for the syntheses of the compounds **1a-c**, **e-g**, **j-l** is presented in scheme 3-1.



Scheme 3-1: General scheme for the synthesis of **1a-c**,**e-g**,**j-l**

3.1.1.1.1 Synthesis of 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride (**3**)

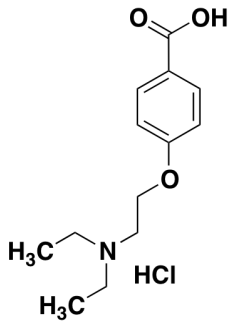


Figure 3-1: Structure of compound **3**

A stirred mixture of methyl 4-hydroxybenzoate (3 g, 0.02 mol), 2-chloro-*N*,*N*-diethylethanamine hydrochloride (5.1 g, 0.03 mol), potassium carbonate (7 g, 0.05 mol), potassium iodide (2 g, 0.01 mol), and acetone (30 ml) was heated at ~70 °C for 4-7 h. On cooling, the solid was removed by filtration and the solvent was evaporated. The residue was dissolved in ethyl acetate (25 ml) and the solution was washed with sodium hydroxide solution (20 ml, 2% w/v) and deionized water (2x20 ml). Removal of the solvent afforded methyl 4-(2-(diethylamino)ethoxy)benzoate as an oil which was dissolved in ethanol (25 ml) and added to a solution of sodium hydroxide (16 g, 0.4 mol) in water (20 ml). The mixture was heated at ~70°C for 2 h. The ethanol was removed in vacuum and the aqueous solution was acidified with hydrochloric acid (12 M) at 5-6 °C. The solid was collected, triturated with water (previously cooled to 5-6 °C), filtered and dried at 55-60 °C in a vacuum oven to give 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride (3.46 g, yield 64%). M.p. 170-175 °C [lit.[39] m.p. 170-172 °C]. ^{1}H NMR (DMSO-d_6): δ 1.23 (t, 6H, 2 x CH_3CH_2), 3.22 (s, 4H, 2 x NCH_2), 3.53 (s, 2H, CH_2CH_2N),

4.42 (s, 2H, CH_2O), 7.08 (d, aryl H), 7.92 (d, 2H, aryl H), 9.80 (s, 1H, HCl). Found C, 56.83; H, 7.72; N, 5.03%. Anal. ($C_{13}H_{20}ClNO_3$) requires C, 57.04; H, 7.36; N, 5.12%.

3.1.1.1.2 Synthesis of ethyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (**1a**)

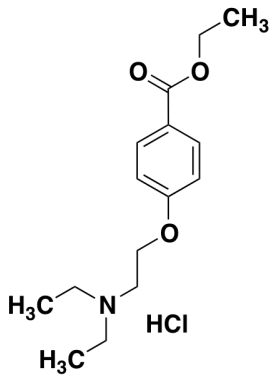


Figure 3-2: Structure of compound **1a**

A stirred mixture of 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride prepared by the literature method[39] (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (1 g, yield 92%). M.p. 144-145 °C. ^{1}H NMR ($CDCl_3$): δ 1.39 (t, 3H, CH_3CH_2), 1.47 (d, 6H, 2 x CH_3CH_2), 3.25 (q, 4H, 2 x CH_3CH_2N), 3.48 (t, 2H, OCH_2CH_2N), 4.35 (q, CH_2O), 4.63 (t, 2H, CH_2COO), 6.93 (m, 2H, Aryl H), 8.02 (m, 2H,

Aryl H), 12.72 (s, 1H, HCl). Found C, 59.82; H, 8.19; N, 5.57%. Anal. ($C_{15}H_{24}ClNO_3$) requires C, 59.69; H, 8.02; N, 4.64%.

3.1.1.1.3 Synthesis of methyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (**1b**)

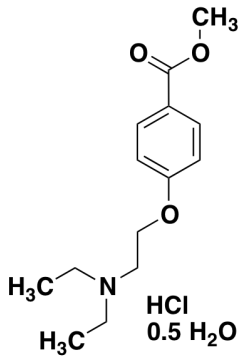


Figure 3-3: Structure of compound **1b**

A stirred mixture of 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride prepared by the literature method[39] (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in methanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from methanol to give methyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (1 g, yield 97%). M.p. 130-135 °C. ^{1}H NMR ($CDCl_3$): δ 1.46 (t, 6H, 2 x CH_3CH_2N), 3.26 (s, 4H, $(CH_2)_2N$), 3.57 (d, 4H, CH_2N), 3.91 (s, 3H, CH_3OCO), 4.63 (s, CH_2O), 6.94 (d, 2H, Aryl H), 8.01 (d, 2H, Aryl H), 12.69 (s, 1H, HCl). Found C, 57.01; H, 7.74; N, 4.66%. Anal. ($C_{14}H_{23}ClNO_{3.5}$) requires C, 56.60; H, 7.75; N, 4.72%.

3.1.1.1.4 Synthesis of isopropyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (**1c**)

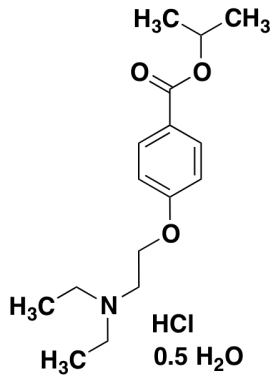


Figure 3-4: Structure of compound **1c**

A stirred mixture of 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride prepared by the literature method[39] (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling the solvent was evaporated. The solid was dissolved in isopropyl alcohol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from isopropyl alcohol to give isopropyl 4-(2-(diethylamino)ethoxy)benzoate hydrochloride (1 g, yield 88%). M.p. 135-140 °C. ^{1}H NMR ($CDCl_3$): δ 1.37 (d, 6H, 2 x CH_3CH), 1.47 (t, 6H, 2 x CH_3CH_2N), 3.26 (q, 4H, 2 x CH_3CH_2N), 3.51 (s, 2H, NCH_2), 4.63 (s, 2H, CH_2O), 5.20 (m, 1H, CH), 6.93 (d, 2H, Aryl H), 8.01 (d, 2H, Aryl H), 12.38 (s, 1H, HCl). Found C, 59.23; H, 8.24; N, 4.25%. Anal. ($C_{16}H_{27}ClNO_{3.5}$) requires C, 59.10; H, 8.30; N, 4.30%.

3.1.1.1.5 Synthesis of ethyl 4-(3-(dimethylamino)propoxy)benzoate hydrochloride (**1i**)

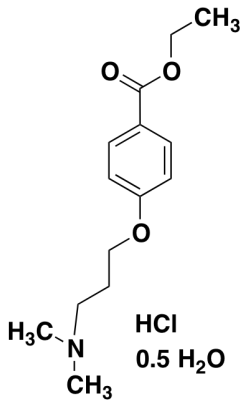


Figure 3-5: Structure of compound **1i**

A stirred mixture of 4-(3-(dimethylamino)propoxy)benzoic acid hydrochloride prepared by the literature method[57] (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 4-(3-(dimethylamino)propoxy)benzoate hydrochloride (1 g, yield 91%). M.p. 150-156 °C. ^{1}H NMR ($CDCl_3$): δ 1.38 (t, 3H, CH_3CH_2), 2.44 (s, 2H, $CH_2CH_2CH_2$), 2.90 (d, 6H, 2 x CH_3N), 3.29 (d, 2H, CH_2N), 4.10 (t, 2H, CH_2O), 4.30 (q, 2H, CH_2COO), 6.80 (d, 2H, aryl H), 8.05 (d, 2H, aryl H), 12.30 (s, 1H, HCl). Found C, 56.29; H, 8.08; N, 4.53%. Anal. ($C_{14}H_{23}ClNO_{3.5}$) requires C, 56.61; H, 7.81; N, 4.86%.

3.1.1.1.6 Synthesis of ethyl 4-(2-(dimethylamino)ethoxy)benzoate hydrochloride (**1e**)

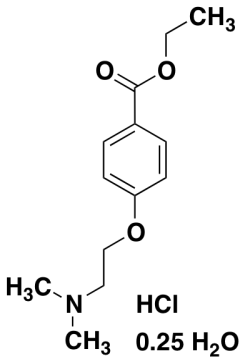


Figure 3-6: Structure of compound **1e**

A stirred mixture of 4-(2-(dimethylamino)ethoxy)benzoic acid hydrochloride prepared by the literature method[57](1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 4-(2-(dimethylamino)ethoxy)benzoate hydrochloride (1 g, 91%). M.p. 140-145 °C. ^{1}H NMR ($CDCl_3$): δ 1.39 (t, 3H, CH_3CH_2), 3.03 (s, 6H, 2 x CH_3N), 3.53 (s, 2H, CH_2N), 4.53 (q, 2H, CH_2COO), 4.69 (s, 2H, CH_2O), 6.90 (d, 2H, aryl H), 8.03 (d, 2H, aryl H), 12.98 (s, 1H, HCl). Found C, 56.08; H, 7.78; N, 4.87%. Anal. ($C_{13}H_{20.5}ClN0_{3.25}$) requires C, 56.06; H, 7.43; N, 5.03%.

3.1.1.1.7 Synthesis of 4-(2-(piperidin-1-yl)ethoxy)benzoic acid hydrochloride (**1f-precurser**)

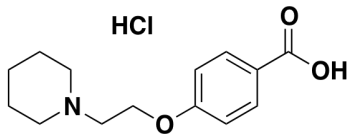


Figure 3-7: Structure of compound **1f-precursor**

A stirred mixture of methyl 4-hydroxybenzoate (6 g, 0.04 mol), 1-(2-chloroethyl)piperidine hydrochloride (10.8 g, 0.06 mol), potassium carbonate (12 g, 0.1 mol), potassium iodide (6 g, 0.04 mol), and acetone (100 ml) was heated at ~70°C for 4-7 h. On cooling, the solid was removed by filtration and the solvent was evaporated. The residue was dissolved in ethyl acetate (50 ml) and the solution was washed with sodium hydroxide solution (40 ml, 2% w/v) and deionized water (2x40 ml). Removal of the solvent afforded methyl 4-(2-(piperidin-1-yl)ethoxy)benzoate as an oil which was dissolved in ethanol (50 ml) and added to a solution of sodium hydroxide (20 g, 0.4 mol) in water (60 ml). The mixture was heated at ~70°C for 2 h. The ethanol was removed in vacuum and the aqueous solution was acidified with hydrochloric acid (12 M) at 5-6 °C. The solid was collected, triturated with water (previously cooled to 5-6 °C), filtered and dried at 55-60 °C in a vacuum oven to give 4-(2-(piperidin-1-yl)ethoxy)benzoic acid hydrochloride (6.83 g, yield 61%). M.p. 259-262 °C. ^{1}H NMR (D_2O): δ 1.37 (t, 1H, piperidine protons), 1.61 (m, 3H, piperidine proton), 1.82 (d, 2H, piperidine protons), 2.90 (m, 2H, OCH_2CH_2N), 3.46 (m, 4H, piperidine protons), 4.33 (q, 2H, OCH_2CH_2N), 6.95 (d, 2H, aryl H), 7.88 (d, 2H, aryl H).

3.1.1.1.8 Synthesis of ethyl 4-(2-(piperidin-1-yl)ethoxy)benzoate hydrochloride (**1f**)

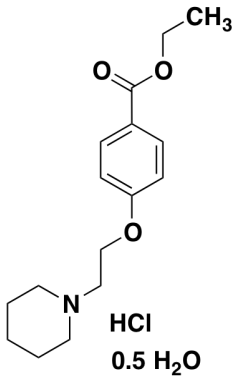


Figure 3-8: Structure of compound **1f**

A stirred mixture of 4-(2-(piperidin-1-yl)ethoxy)benzoic acid hydrochloride (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 4-(2-(piperidin-1-yl)ethoxy)benzoate hydrochloride (1 g, yield 91%). M.p. 170-173 °C. ^{1}H NMR ($CDCl_3$): δ 1.39 (t, 3H, CH_3CH_2), 1.90 (t, 4H, piperidine proton), 2.27 (q, 2H, piperidine protons), 2.80 (q, 2H, piperidine protons), 3.44 (s, 2H, piperidine protons), 3.67 (d, 2H, OCH_2CH_2N), 4.35 (q, 2H, $COOCH_2CH_3$), 4.66 (s, 2H, OCH_2CH_2N), 6.93 (d, 2H, aryl H), 8.02 (d, 2H, aryl H), 12.42 (s, 1H, HCl). Found C, 59.62; H, 8.06; N, 4.16%. Anal. ($C_{16}H_{25}ClNO_{3.5}$) requires C, 59.47; H, 7.81; N, 4.33%.

3.1.1.1.9 Synthesis of 4-(2-morpholin-4-ylethoxy)benzoic acid hydrochloride (**1g-precursor**)

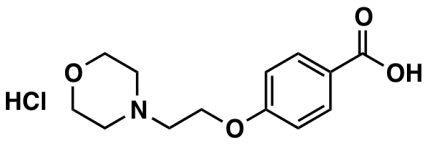


Figure 3-9: Structure of compound **1g-precursor**

A stirred mixture of methyl 4-hydroxybenzoate (5 g, 0.03 mol), 4-(2-chloroethyl)morpholine (9.2 g, 0.05 mol), potassium carbonate (10.2 g, 0.07 mol), potassium iodide (2 g, 0.012 mol), and acetone (30 ml) was heated at ~70°C for 4-7 h. On cooling, the solid was removed by filtration and the solvent was evaporated. The residue was dissolved in ethyl acetate (25 ml) and the solution was washed with sodium hydroxide solution (20 ml, 2% w/v) and deionized water (2x20 ml). Removal of the solvent afforded methyl 4-(2-morpholin-4-ylethoxy)benzoate as an oil which was dissolved in ethanol (25 ml) and added to a solution of sodium hydroxide (8 g, 0.2 mol) in water (20 ml). The mixture was heated at ~70°C for 2 h. The ethanol was removed in vacuum and the aqueous solution was acidified with hydrochloric acid (12 M) at 5-6 °C. The solid was collected, triturated with water (previously cooled to 5-6 °C), filtered and dried at 55-60 °C in a vacuum oven to give 4-(2-morpholin-4-ylethoxy)benzoic acid hydrochloride (6.58 g, yield 85%). M.p. 221-225 °C. ^{1}H NMR (D_2O): δ 3.21 (q, 2H, morpholine protons), 3.47 (m, 2H, morpholine protons), 3.56 (t, 2H, OCH_2CH_2N), 3.71 (q, 2H, morpholine protons), 4.01 (s, 2H, morpholine protons), 4.36 (t, 2H, OCH_2CH_2N), 6.96 (d, 2H, aryl H), 7.87 (d, 2H, aryl H).

3.1.1.1.10 Synthesis of ethyl 4-(2-(morpholin-4-yl)ethoxy)benzoate hydrochloride (**1g**)

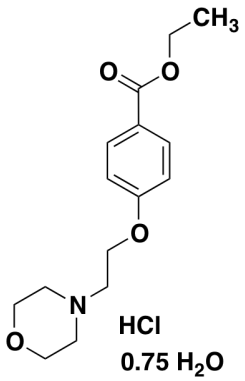


Figure 3-10: Structure of compound **1g**

A stirred mixture of 4-(2-(morpholin-4-yl)ethoxy)benzoic acid hydrochloride (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 4-(2-(morpholin-4-yl)ethoxy)benzoate hydrochloride (1 g, yield 91%). M.p. 190-194 °C. ^{1}H NMR ($CDCl_3$): δ 1.40 (t, 3H, CH_3CH_2), 3.07 (q, 2H, CH_2COO), 3.48 (s, 2H, morpholine protons), 3.58 (d, 2H, morpholine protons), 4.02 (t, 2H, morpholine protons), 4.29 (d, 2H, morpholine protons), 4.43 (q, 2H, NCH_2CH_2O), 4.70 (s, 2H, OCH_2CH_2N), 6.90 (d, 2H, aryl H), 8.00 (d, 2H, aryl H), 12.72 (s, 1H, HCl). Found C, 54.68; H, 7.07; N, 4.06%. Anal. ($C_{15}H_{23.5}ClNO_{4.75}$) requires C, 54.66; H, 7.19; N, 4.25%.

3.1.1.1.11 Synthesis of (4-(2-(diethylaminoethoxy)phenyl)(piperidin-1-yl)methanone hydrochloride (**1d**)

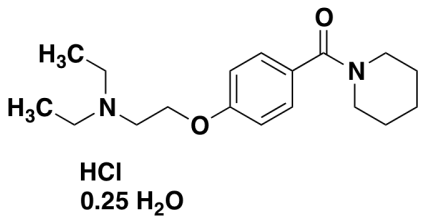


Figure 3-11: Structure of compound **1d**

A stirred mixture of 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride prepared by the literature method[39] (1 g, 0.0036 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated and the solid dissolved in 1,2 dichloroethane (10 ml). A mixture of piperidine (0.7 g, 0.01 mol) and 1,2 dichloroethane (10 ml) was added dropwise to the acid chloride solution surrounded by an ice bath and stirred overnight. The solvent was removed and the residue basified with potassium carbonate (10%, 30 ml, w/v). The compound was extracted in ethyl acetate and washed with water. Hydrochloric acid gas was passed into the solution, the resultant solid was filtered, recrystallized from tetrahydrofuran (THF) to give (4-(2-(diethylamino)ethoxy)phenyl)(piperidin-1-yl)methanone hydrochloride (0.84 g, yield 68%). M.p. 155-158 °C. ^{1}H NMR (DMSO-d_6): δ 1.24 (t, 6H, 2 x CH_3), 1.50 (s, 6H, piperidine protons), 1.61 (d, 2H, CH_2N), 3.2 (s, 4H, 2 x N*C*$H_2$$CH_3$), 3.50 (s, 4H, piperidine protons), 4.40 (s, 2H, O*C*H_2), 7.03 (d, 2H, aryl H), 7.36 (d, 2H, aryl H), 10.30

(s, 2H, HCl). Found C, 62.50; H, 8.51; N, 8.0%. Anal. ($C_{18}H_{29.5}ClN_2O_{2.25}$) requires C, 62.50; H, 9.00; N, 8.11%.

3.1.1.1.12 Synthesis of ethyl 4-(isopentyloxy)benzoate (**1h**)

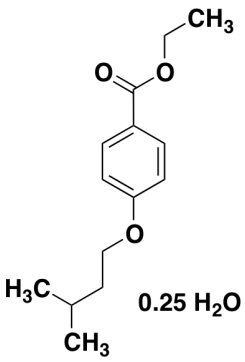


Figure 3-12: Structure of compound **1h**

A stirred mixture of 4-(isopentyloxy)benzoic acid prepared by the literature method[51] (1 g, 0.0048 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid dried at 55-60 °C in a vacuum oven to give ethyl 4-(isopentyloxy)benzoate (0.9 g, yield 79%). ^{1}H NMR ($CDCl_3$): δ 0.98 (d, 6H, 2 x *CH*$_3$CH), 1.39 (t, 3H, *CH*$_3$CH$_2$COO), 1.70 (q, 2H, CH*CH*$_2$), 1.82 (sextet, 1H, CH), 4.04 (t, 2H, OCH_2), 4.34 (q, 2H, CH_3*CH*$_2$OCO), 6.91 (d, 2H, aryl H), 8.00 (d, 2H, aryl H). Found C, 71.09; H, 8.77%. Anal. ($C_{14}H_{20.5}O_{3.25}$) requires C, 71.41; H, 9.11%.

3.1.1.1.13 Synthesis of methyl 4-(isopentyloxy)benzoate (**1m**)

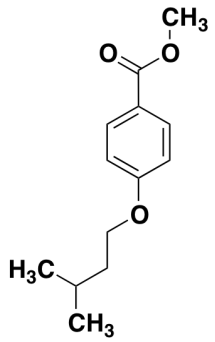


Figure 3-13: Structure of compound **1m**

A stirred mixture of methyl 4-hydroxybenzoate (5 g, 0.03 mol), 1-bromo-3-methylbutane (6.5 g, 0.04 mol), potassium carbonate (7.7 g, 0.06 mol), and potassium iodide (2 g, 0.01 mol) was heated at ~70°C for 4-7 h. On cooling, the solid was removed by filtration and the solvent was evaporated. The residue was dissolved in ethyl acetate (25 ml) and the solution was washed with sodium hydroxide solution (20 ml, 2% w/v) and deionized water (2x20 ml). Removal of the solvent afforded methyl 4-(isopentyloxy)benzoate as an oil. (5.45 g, yield 75%). ^{1}H NMR ($CDCl_3$): δ 0.97 (d, 6H, *CH*$_3$CH), 1.68 (q, 2H, *CH*$_2$CH), 1.81 (m, 1H, CH), 3.89 (s, 3H, *CH*$_3$OCO), 4.03 (t, 2H, OCH_2), 6.90 (d, 2H, aryl H), 7.98 (d, 2H, aryl H). Found C, 70.65; H, 8.48 Anal. ($C_{13}H_{18}O_3$) requires C, 70.24; H, 8.16%.

3.1.1.1.14 Synthesis of ethyl 3-(2-(dimethylamino)ethoxy)benzoate hydrochloride (**1l**)

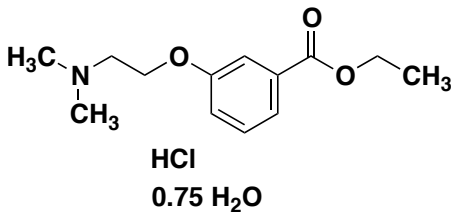


Figure 3-14: Structure of compound **1l**

A stirred mixture of 3-(2-(dimethylamino)ethoxy)benzoic acid hydrochloride (commercially available) (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven and recrystallized from ethanol to give ethyl 3-(2-(dimethylamino)ethoxy)benzoate hydrochloride (1.04 g, yield 95%). M.p. 140-144 °C. ^{1}H NMR ($CDCl_3$): δ 1.40 (t, 3H, CH_3CH_2), 2.97 (d, 6H, 2 x CH_3), 3.53 (s, 2H, NCH_2), 4.38 (t, 2H, CH_2OCO), 4.42 (s, 2H, OCH_2), 7.14 (d, 1H, aryl H), 7.38 (s, 1H, aryl H), 7.56 (s, 1H, aryl H), 7.72 (d, 1H, aryl H), 13.00 (s, 1H, HCl). Found C, 54.09; H, 7.26; N, 4.63%. Anal. ($C_{13}H_{21.5}ClNO_{3.75}$) requires C, 54.31; H, 7.54; N, 4.87%..

3.1.1.1.15 Synthesis of ethyl 2-(2-(diethylamino)ethoxy)benzoate hydrochloride (**1j**)

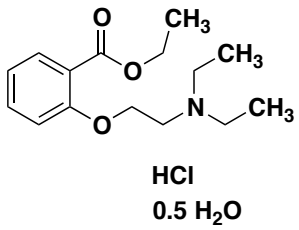


Figure 3-15: Structure of compound **1j**

A stirred mixture of 2-(2-(diethylamino)ethoxy)benzoic acid hydrochloride (commercially available) (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven to give ethyl 2-(2-(diethylamino)ethoxy)benzoate hydrochloride (0.27 g, yield 24%). ^{1}H NMR ($CDCl_3$): δ 1.40 (t, 3H, CH_3CH_2), 1.49 (s, 6H, 2 x CH_2CH_3), 3.30 (s, 4H, 2 x CH_3CH_2), 3.50 (s, 2H, CH_2N), 4.37 (q, 2H, CH_2CH_2O), 4.60 (s, 2H, $COOCH_2$), 7.12 (d, 1H, aryl H), 7.38 (t, 1H, aryl H), 7.55 (s, 1H, aryl H), 7.71 (d, 1H, aryl H), 12.55 (s, 1H, HCl). Found C, 57.87; H, 8.13; N, 4.33%. Anal. ($C_{15}H_{25}ClNO_{3.5}$) requires C, 57.91; H, 8.10; N, 4.50%.

3.1.1.1.16 Synthesis of ethyl 3-(2-(diethylamino)ethoxy)benzoate hydrochloride (**1k**)

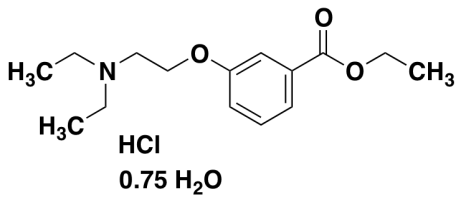


Figure 3-16: Structure of compound **1k**

A stirred mixture of 3-(2-(diethylamino)ethoxy)benzoic acid hydrochloride (commercially available) (1 g, 0.004 mol) and thionyl chloride (20 ml) was heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated. The solid was dissolved in ethanol (20 ml) and the solution was heated at ~70°C for 3-5 h. On cooling, the solvent was evaporated and the solid was dried at 55-60 °C in a vacuum oven to give ethyl 3-(2-(diethylamino)ethoxy)benzoate hydrochloride (1.12 g, yield 93%). M.p. 110-111 °C. ^{1}H NMR (DMSO-d_6): δ 1.24 (t, 6H, 2 x CH_3CH_2), 1.31 (t, 3H, $COOCH_2CH_3$), 3.21 (q, 4H, 2 x CH_2N), 3.51 (d, 2H, CH_2N), 4.30 (q, 2H, CH_2CH_2O), 4.42 (t, 2H, $COOCH_2$), 7.29 (d, 1H, aryl H), 7.48 (t, 2H, aryl H), 7.60 (d, 1H, aryl H), 10.22 (s, 1H, HCl). Found C, 56.75; H, 8.19; N, 4.12%. Anal. ($C_{15}H_{25.5}ClNO_{3.75}$) requires C, 57.08; H, 8.07; N, 4.44%.

3.1.1.2 Synthesis of Series **2**

3.1.1.2.1 Synthesis of (3*E*,5*E*)-3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one (**2a**)

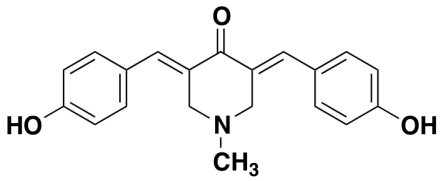


Figure 3-17: Structure of compound **2a**

A stirred mixture of 4-hydroxybenzaldehyde (6.9 g, 0.06 mol), 1-methylpiperidin-4-one (3.22 g, 0.03 mol), acetic acid (70 ml), and ethanolic HCl (30 ml) were heated at ~70°C overnight. The solid was filtered and stirred with potassium carbonate solution (20%, 100 ml, w/v) for two hours. The solid was filtered and dried at 55-60 °C in a vacuum oven to give (3*E*,5*E*)-3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one and recrystallized from isopropyl ether to give 1.12 g (yield 12%). M.p. 245-249 °C. [lit.[58] m.p. 197-199 °C]. ^{1}H NMR (DMSO-d_6): δ 2.40 (s, 3H, NCH_3), 3.65 (s, 4H, 2 x CH_2NCH_3), 6.73 (d, 4H, 2 x aryl H), 7.26 (d, 4H, 2 x aryl H), 7.47 (s, 2H, 2 x CH).

3.1.1.2.2 Synthesis of (3*E*,5*E*) 3,5-bis{4-[4-(2-diethylaminoethoxy)phenyl-carbonyloxy]benzylidene}-1-methyl-4-piperidone (**2b**)

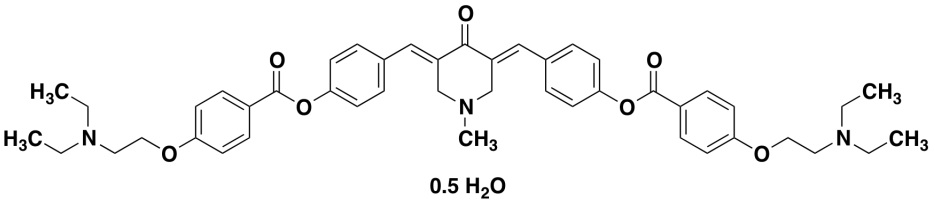


Figure 3-18: Structure of compound **2b**

4-(2-(Diethylamino)ethoxy)benzoyl chloride hydrochloride was prepared by adding thionyl chloride (25 ml) to 4-(2-(diethylamino)ethoxy)benzoic acid hydrochloride (1g) and heated at ~70°C for 2-3 h. On cooling, solvent was evaporated to yield 4-(2-(diethylamino)ethoxy)benzoyl chloride hydrochloride which was used without further purification.

Triethylamine (2.18 g, 0.02 mol) in 1,2-dichloroethane (10 ml) was added dropwise to a stirred suspension of (3*E*,5*E*)-3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one[58] (1 g, 0.003 mol) and 4-(2-(diethylamino)ethoxy)benzoyl chloride hydrochloride (2.4 g, 0.01 mol) in 1,2-dichloroethane (30 ml) at ~5 °C for a period of 30 min under nitrogen. The reaction mixture was stirred at room temperature overnight. The mixture was filtered and the solvent evaporated. The residue was stirred in potassium carbonate solution (70 ml, 7% w/v) for two hours. The solid obtained was filtered, dried in a vacuum oven at 50° C, and crystallized from ethyl acetate to yield the pure compound (1.6 g, yield 70%). M.p. 122-125 °C. Yield 70%. ^{1}H NMR ($CDCl_3$): δ

1.10 (d, 12H, 4 X CH_3CH_2N), 2.52 (s, 3H, CH_3N), 2.65 (m, 8H, 4 x CH_3CH_2N), 2.93 (t, 4H, 2 x OCH_2CH_2N), 3.81 (s, 4H, 2 x CH_3NCH_2), 4.15 (t, 4H, 2 x OCH_2CH_2N), 6.99 (t, 4H, aryl H), 7.29 (d, 4H, aryl H), 7.48 (d, 4H, aryl H), 7.85 (s, 2H, 2 x CH), 8.16 (d, 4H, aryl H). ^{13}C NMR ($CDCl_3$): δ 186.812, 164.720, 163.438, 151.507, 135.576, 133.142, 132.843, 132.378, 132.326, 131.646, 122.084, 121.440, 121.163, 114.465, 114.389, 77.294, 77.040, 76.786, 67.050, 57.082, 51.578, 47.945, 45.924, 30.979, 11.884. MS *m/z* 760.4 [M + H]. Found C, 72.02; H, 6.96; N, 5.33%. Anal. ($C_{46}H_{54}N_3O_{7.5}$) requires C, 71.79; H, 7.02; N, 5.46%.

3.1.1.2.3 Synthesis of (3*E*,5*E*) 3,5-bis{4-[4-(2-piperidin-1-ylethoxy)phenyl-carbonyloxy]benzylidene}-1-methyl-4-piperidone (**2c**)

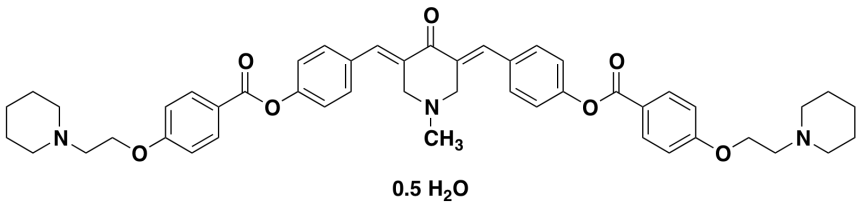


Figure 3-19: Structure of compound **2c**

4-(2-(Piperidin-1-yl)ethoxy)benzoyl chloride hydrochloride was prepared by adding thionyl chloride (25 ml) to 4-(2-(piperidin-1-yl)ethoxy)benzoic acid hydrochloride and heated at ~70°C for 2-3 h. On cooling, the solvent was evaporated to yield 4-(2-(piperidin-1-yl)ethoxy)benzoyl chloride hydrochloride which was used without further purification.

Triethylamine (0.8 g, 0.01 mol) in chloroform (10 ml) was added dropwise to a stirred suspension of (3*E*,5*E*)-3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one[58] (1 g, 0.003 mol) and 4-(2-(piperidin-1-yl)ethoxy)benzoyl chloride hydrochloride (2.4 g, 0.01 mol) in 1,2-dichloroethane (30 ml) at ~5 °C for a period of 30 min under nitrogen. The reaction mixture was stirred at room temperature overnight. The mixture was filtered and the solvent evaporated. The residue was stirred in potassium carbonate solution (70 ml, 7% w/v) for two hours. The solid obtained was filtered, dried in a vacuum oven at 50° C to yield the pure compound (580 mg, yield 25%). M.p. 135-137 °C. Yield 24 %. ^{1}H NMR ($CDCl_3$): δ 1.42 (s, 3H, CH_3), 1.62 (m, 10H, piperidine protons), 2.52 (d, 10H, piperidine protons), 2.82 (t, 4H, 2 x CH_2CH_2N), 3.81 (s, 4H, 2 x CH_2NCH_3), 4.21 (t, 4H, OCH_2CH_2N), 7.11 (d, 4H, aryl H), 7.31 (s, 4H, aryl H), 7.43 (m, 4H, aryl H), 7.85 (s, 2H, 2 x CH), 8.16 (d, 4H, aryl H). ^{13}C NMR ($CDCl_3$): δ 186.811, 164.708, 163.391, 151.500, 135.569, 133.148, 132.848, 132.378, 131.644, 122.081, 121.476, 114.505, 77.292, 77.038, 76.784, 66.421, 57.763, 57.082, 55.167, 45.923, 30.974, 25.977, 24.180, 1.044. MS *m/z* 784.6 [M + H]. Found C, 72.78; H, 7.00; N, 5.03%. Anal. ($C_{48}H_{54}N_3O_{7.5}$) requires C, 72.64; H, 6.8; N, 5.30%.

3.1.1.2.4 Synthesis of (3*E*,5*E*) 3,5-bis(4-(4-(2-(4-morpholino)ethoxy)phenyl carbonyloxy)benzylidene)-1-methyl-4-piperidone (**2d**)

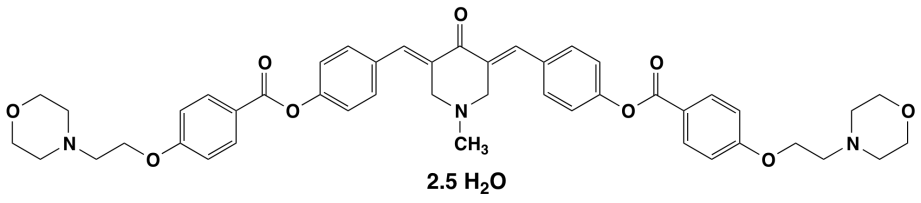


Figure 3-20: Structure of compound **2d**

4-(2-Morpholin-4-ylethoxy)benzoyl chloride hydrochloride was prepared by adding thionyl chloride (25 ml) to 4-(2-morpholin-4-ylethoxy)benzoic acid hydrochloride and heated at ~70°C for 2-3 h. On cooling, solvent was evaporated to yield 4-(2-morpholin-4-ylethoxy)benzoyl chloride hydrochloride which was used without further purification.

Triethylamine (0.9 g, 0.01 mol) in 1,2-dichloroethane (10 ml) was added dropwise to a stirred suspension of (3*E*,5*E*)-3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one[58] (0.6 g, 0.002 mol) and 4-(2-morpholin-4-ylethoxy)benzoyl chloride hydrochloride (1.25 g, 0.004 mol) in 1,2-dichloroethane (30 ml) at ~5 °C for a period of 30 min under nitrogen. The reaction mixture was stirred at room temperature overnight. The mixture was filtered and the solvent evaporated. The residue was stirred in potassium carbonate solution (70 ml, 7% w/v) for two hours. The solid obtained was filtered, dried in a vacuum oven at 50° C, and crystallized from ethanol to yield the pure compound (0.93 g, yield 68%). M.p. 160-165 °C. Yield 68%. ^{1}H NMR ($CDCl_3$): δ 1.64 (s, 4H, 2 x NCH_2), 2.52 (d, 3H, CH_3N), 2.63 (s, 8H, morpholinyl protons), 2.87 (t, 4H, 2 x CH_2NCH_3), 3.77 (q, 8H, morpholinyl protons), 4.22 (t, 4H, 2 x CH_2O), 7.02 (d, 4H, aryl H), 7.31 (s, 4H, aryl H), 7.48 (d, 4H, aryl H), 7.85 (s, 2H, 2 x CH), 8.17 (d, 4H, aryl H). ^{13}C NMR ($CDCl_3$): δ 186.789, 164.656, 163.198, 151.471, 135.567, 133.143, 132.871, 132.413, 131.650, 122.069, 121.682, 111.476, 77.296, 77.043, 76.788, 66.931, 66.165, 57.463, 57.072, 54.157, 45.916, 1.046. MS *m/z* 788.6 [M + H]. Found C, 66.34; H, 6.20; N, 5.00%. Anal. ($C_{46}H_{54}N_3O_{11.5}$) requires C, 66.27; H, 6.48; N, 5.04%.

3.2 Bioevaluations

3.2.1 Materials and Methods

3.2.1.1 Cytotoxic assays

Series **1** and **2** compounds were evaluated against L1210, Molt 4/C8, CEM, and HeLa cells using a previously reported procedure.[59] Different concentrations of the compounds were incubated with the appropriate cell line in RPMI 1640 medium at 37 ˚C for 72h (Molt 4/C8 and CEM) or 48h (HeLa and L1210). Cell numbers were determined using a Coulter counter. The IC_{50} value given is the concentration required to inhibit cell proliferation by 50%. Data are expressed as the mean ±SD from the dose–response curves of at least three independent experiments. Melphalan and curcumin were employed under the same conditions as positive controls.

Compounds in series **1** and **2** were evaluated at a concentration of 5 or 1 μM against JURKAT, BJAB, Nalm-6, EL-4, BW5147, LNcAP, and DU145, RAJI B, Ramos, HUT-102, Molt-3, Sup-T1, HT-29, COLO 205, LAPC4, HCC70, and Hs27 cell lines. A solution of each compound in series **1** and **2** in DMSO-d_6 was added to the cells grown in the appropriate (RPMI or DMEM) media, and incubated for 22 h at 37 ˚C. The average cytotoxicity of three independent experiments was obtained by observing the plasma membrane disruption using flow cytometry with propidium iodide.[60]

Series **1** and **2** compounds were also evaluated against human oral non-malignant and neoplastic cell lines HGF, HPC, HPLF, HSC-2, HSC-3, HSC-4, and HL-60 following a literature method except for the incubation time (48h). Various concentrations (maximum of 400 μM) of each compound as well as untreated cells were

added to cultured cells and incubated for 48h at 37 °C. The cytotoxic concentration (CC_{50}) values were determined from dose-response curves and the figures represent the mean from duplicated experiments.[61]

3.2.1.2 MDR-reversal assay

Series **2** compounds were added to a transfected murine lymphoma 5178Y cells with the mdr-1 gene following a literature procedure[62] and cultured in the presence of colchicine in order to maintain the expression of the MDR phenotype. Solutions of two concentrations (5 and 50 μg/mL) of the compounds in dimethylsulfoxide were added to mdr-1 transfected and the L5178 parenteral cells at room temperature and incubated for 10 min. Then, rhodamine 123 solution was added and the cells were incubated for a further 20 min at 37 °C. The fluorescence of the parental and mdr-1 treated and untreated cells was measured with a Becton-Dickinson FACScan flow cytometer. Verapamil was used as a positive control. Fluorescence activity ratio (FAR) for each compound were calculated from the following equation:

$$FAR = \frac{\text{MDR treated / MDR control}}{\text{parental treated / parental control}}$$

Equation 3-1: The fluorescence activity ratio equation

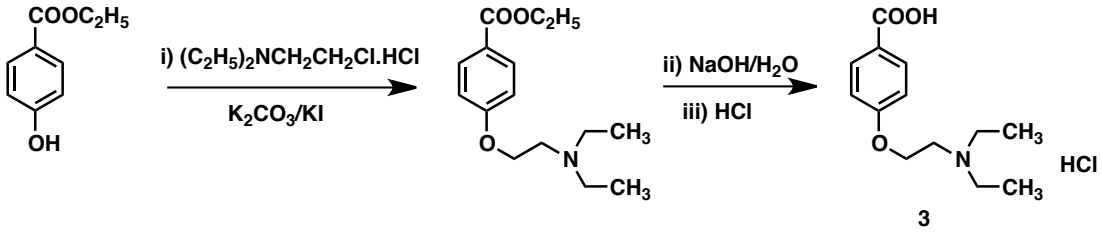
Verapamil was used as a positive control using an intermediate concentration between 5 and 50 μg/mL, namely 10 μg/mL.[63]

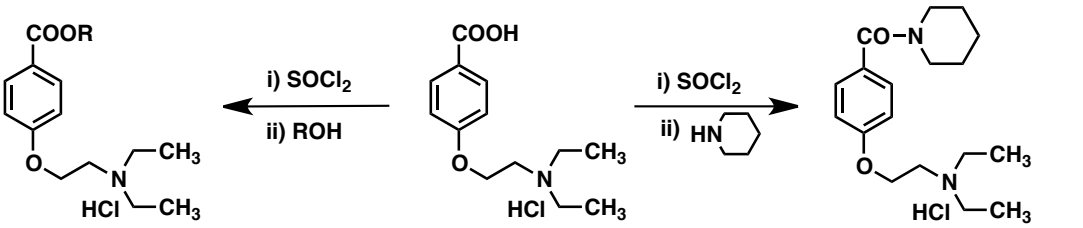
CHAPTER 4

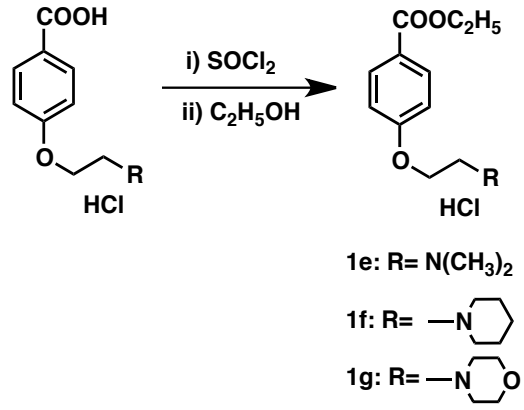
4. Results and Discussion

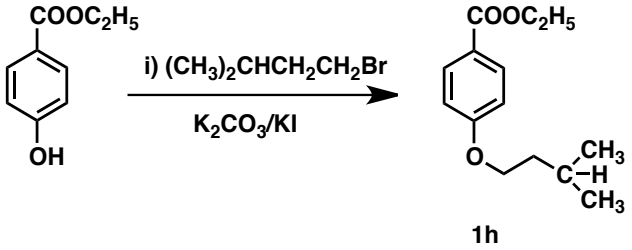
4.1 Results

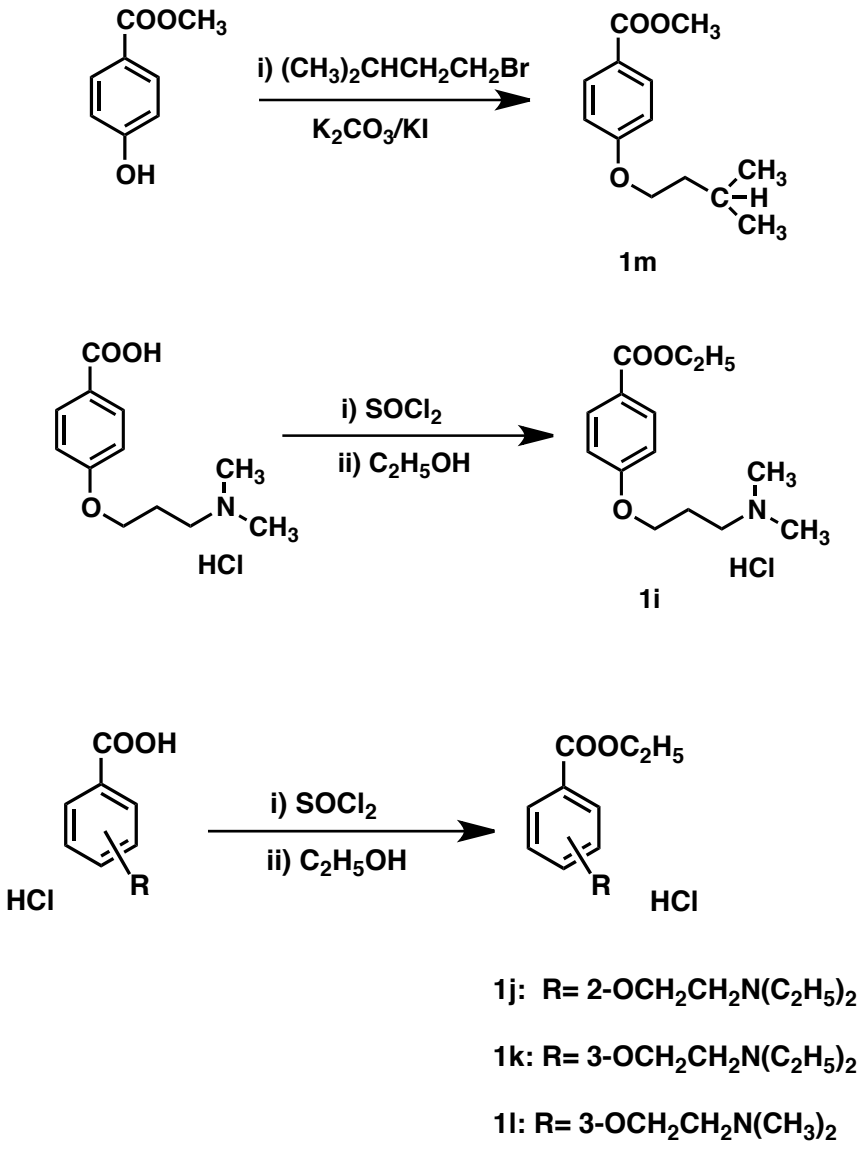
Series **1** and **2** were synthesized successfully. The elemental analyses (for carbon and hydrogen for all compounds, and nitrogen when present in the molecules) were within 0.4% of the calculated values. In series **2**, the *E* stereochemistry was confirmed using ^{1}H NMR spectroscopy. The auxiliary binders **1** were prepared by the sequence of reactions as illustrated in Scheme 4-1. The synthesis of **3** was previously reported.[39] Ethyl, methyl, and isopropyl esters **1a-c**,**e-g**,**i-l** were prepared by reacting 2,3, or 4-substituted benzoic acids with thionyl chloride to afford the corresponding acid chlorides. Subsequent condensation was followed with ethyl, methyl, and isopropyl alcohols to afford the corresponding esters **1a-c**,**e-g**,**i-l**. Compound **1d** was prepared by different method. The acid chloride of compound **3** was prepared by the reaction of **3** with thionyl chloride, followed by a reaction with piperidine under cooling which afforded **1d**. The alkaline catalyzed condensation of ethyl and methyl 4-hydroxy benzoate and isopentyl bromide under basic conditions afforded the ethyl and methyl esters **1h** and **1m**. The synthetic endeavor for series **1** is presented in Scheme 4-1. Series **2** was prepared by the condensation of 4-hydroxybenzaldehyde and N-methyl-4-piperidone under acidic conditions to afford 3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one **2a**. For **2b-d**, the reaction took place by adding the appropriate aroyl chlorides in series **1** to 3,5-bis(4-hydroxybenzylidene)-1-methylpiperidin-4-one under basic conditions. This endeavor is indicated in Scheme 4-2.


COOC2H5
OH
i) (C2H5)2NCH2CH2Cl.HCl
K2CO3/KI
COOC2H5
O
N
CH3
CH3
ii) NaOH/H2O
iii) HCl
COOH
HCl
3

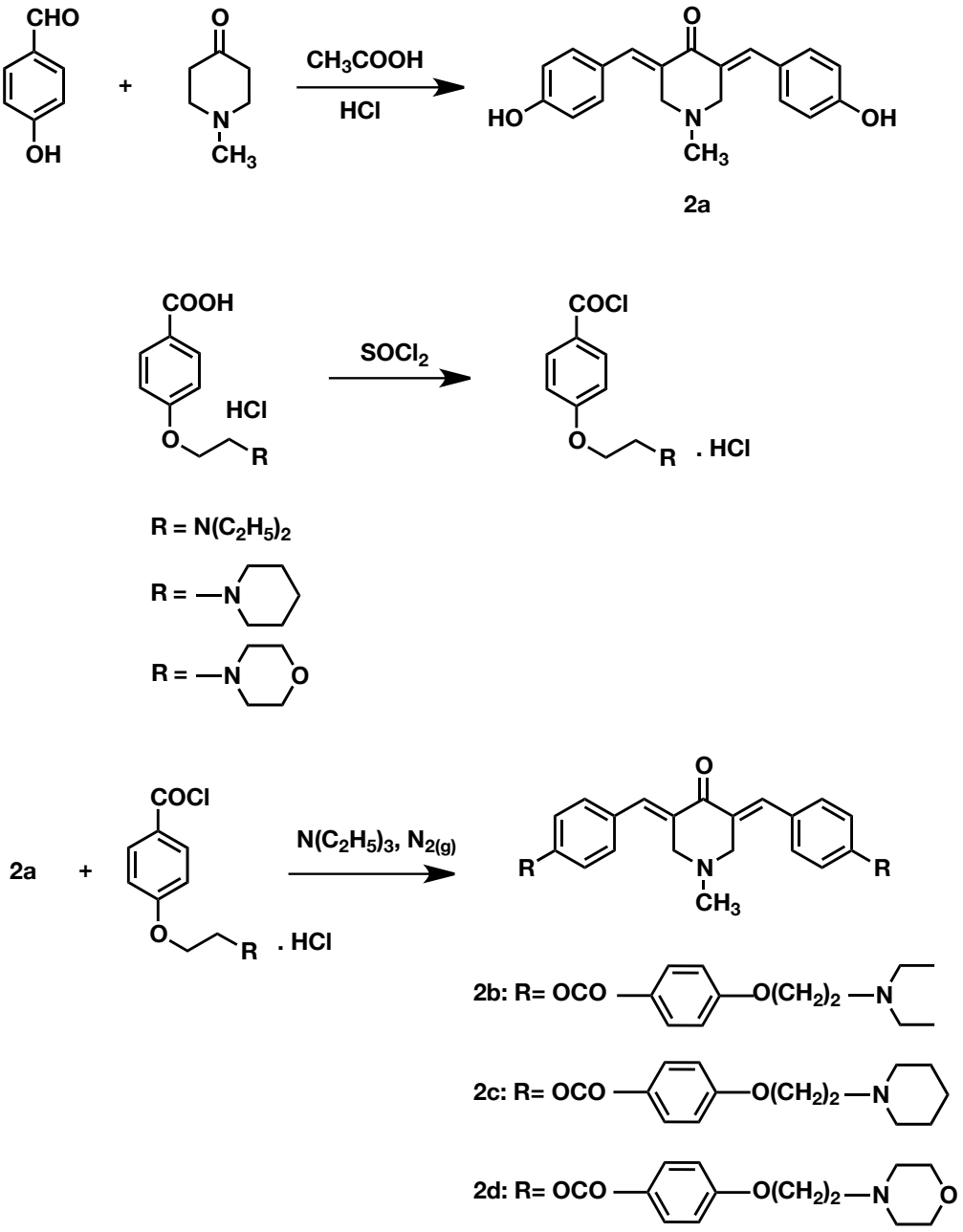

COOR
i) SOCl2
ii) ROH
COOH
i) SOCl2
ii) HN
CO–N
HCl
1a: R= C2H5
1b: R= CH3
1c: R= CH(CH3)2
3
1d


COOH
i) SOCl2
ii) C2H5OH
COOC2H5
O
R
HCl
1e: R= N(CH3)2
1f: R=
1g: R=
N
O


COOC2H5
OH
i) (CH3)2CHCH2CH2Br
K2CO3/KI
O
C–H
CH3
CH3
1h



Scheme 4-1: Synthetic endeavors of analogs **1a-m**

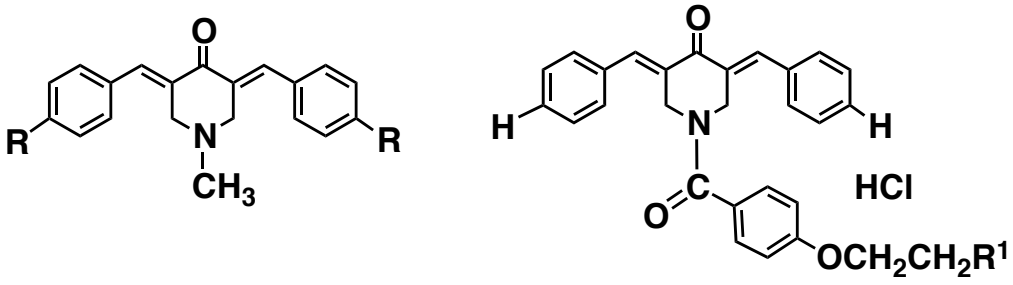


Scheme 4-2: Synthetic endeavors of analogs **2a-d**

All of the compounds in series **1** and **2** were evaluated towards various human and murine transformed cells in order to determine their cytotoxic effects. As well, normal

cells were also involved in the biological evaluations to determine whether these compounds have greater toxicity to neoplasms than non-malignant cells. Melphalan and curcumin were used as the standard anticancer drugs in these assays.

Specifically the following bioevaluations were undertaken. All of the compounds in series **1** and **2** were evaluated against murine L1210 lymphocytic leukemia cells and human Molt4/C8 and CEM T-lymphocytes as well as human HeLa cervix carcinoma cells. Cytotoxic potencies are expressed as IC_{50} values and the biodata are presented in Tables 4-1 and 4-2. The clogP values for the compounds in series **1** and **2** were obtained from a commercial software package and these results are indicated in the same tables as well. The IC_{50} values of previously reported compounds from this laboratory **4a-c** and **5a-d** (Figure 4-1) are also indicated in the Table 4-2 for comparison purposes.[39,64]



4a: R= H
4b: R= CH_3
4c: R= OCH_3

5a: R^1= $N(CH_3)_2$
5b: R^1= $N(C_2H_5)_2$
5c: R^1= 
5d: R^1= 

Figure 4-1: Structures of series **4** and **5**

Table 4-1: Evaluation of series **1a-m** and analog **3** against Molt4/C8, CEM, L1210, and HeLa cancer cells[59]

Compd	IC_{50} (μM)[a]				
	L1210	Molt4/C8	CEM	HeLa	clogP [b]
1a	193 ± 49	> 500	225 ± 49	246 ± 64	3.97
1b	280 ± 60	> 500	276 ± 51	278 ± 60	3.44
1c	102 ± 19	205 ± 2	192 ± 31	147 ± 45	4.28
1d	365 ± 166	> 500	355 ± 123	354 ± 56	3.18
1e	232 ± 33	338 ± 35	229 ± 28	≥ 500	2.91
1f	170 ± 3	220 ± 2	173 ± 10	206 ± 2	4.15
1g	358 ± 30	441 ± 83	286 ± 1	222 ± 19	2.93
1h	105 ± 43	51.8 ± 1.6	69.4 ± 17.3	154 ± 67	5.86
1i	129 ± 18	224 ± 5	174 ± 21	232 ± 12	3.26
1j	340 ± 57	> 500	248 ± 25	308 ± 182	3.73
1k	216 ± 0	176 ± 15	180 ± 20	187 ± 16	3.97
1l	244 ± 30	400 ± 47	232 ± 18	294 ± 101	2.91
1m	208 ± 8	220 ± 3	191 ± 21	292 ± 90	4.27
3	>500	>500	>500	>500	0.91

[a] The IC_{50} value is the concentration of a compound required to inhibit the growth of the cells by 50%.

[b] The clogP values were obtained from ChemDraw software.

Table 4-2: Evaluation of series **2**, **4**, and **5** against Molt4/C8, CEM, L1210, and HeLa cancer cells[59]

Compd	IC_{50} (μM)[a]				
	L1210	Molt4/C8	CEM	HeLa	clogP[b]
2a	55.7 ± 20.2	18.9 ± 7.8	19.2 ± 12.6	18.9 ± 7.2	2.92
2b	10.5 ± 1.0	43.6 ± 0.6	27.8 ± 17.8	20.2 ±11.7	9.75
2c	6.05 ± 1.94	22.3 ± 13.8	16.5 ± 9.3	8.21 ±0.85	10.22
2d	7.82 ± 4.03	12.0 ± 0.6	30.8 ± 17.3	10.5 ± 3.6	7.80
4a	8.77 ± 0.28	1.98 ± 0.27	3.32 ± 2.30	N/A[c]	3.39
4b	305 ± 10	277 ± 6	233 ± 27	N/A[c]	4.36
4c	281 ± 15	230 ± 1	172 ± 6	N/A[c]	3.13
5a[d]	10.2 ± 0.8	0.58 ± 0.09	1.24 ± 0.57	N/A[c]	5.18
5b[d]	8.23 ± 0.43	1.28 ± 0.15	1.65 ± 0.15	N/A[c]	6.24
5c[d]	8.40 ± 0.22	1.48 ± 0.12	1.16 ± 0.64	N/A[c]	6.41
5d[d]	222 ± 13	8.42 ± 0.16	6.84 ± 0.09	N/A[c]	5.20
Curcumin	15.1 ± 1.6	6.46 ± 1.41	8.16 ± 1.66	21.2 ±16.1	2.25
Melphalan	4.85 ± 0.87	2.81 ± 0.33	1.44 ± 0.20	1.70 ±0.44	- 0.207

[a] The IC_{50} value is the concentration of a compound required to inhibit the growth of the cells by 50%.

[b] The clogP values were obtained from ChemDraw software.

[c] N/A means that the result is not available.

[d] The data for the IC_{50} values were taken from reference 46 and are reproduced by permission of the copyright owner.

Some of compounds in series **1** were screened against human Jurkat, EL-4, BW5147, BJAB, Nalm-6 lymphocytic leukemia, LNcAP and DU145 prostate carcinoma cells at a concentration of 5 μM. The biological activity was expressed as the growth inhibition percentages. These data are presented in Table 4-3.

Table 4-3: Evaluation of **1e-g,i-l** and **3** against various cancer cells[60]

Compd	µM	% Growth Inhibition						
		JURKAT	BJAB	Nalm-6	EL-4	BW5147	LNcAP	DU145
1e	5	0.4 ± 0.7	3.9 ± 6.1	1.1 ± 1.0	1.7 ± 3.0	0.0 ± 0.0	1.4 ± 2.4	2.3 ± 2.0
1f	5	0.3 ± 0.3	0.4 ± 0.7	1.9 ± 1.7	0.3 ± 0.4	0.5 ± 0.5	1.3 ± 2.2	6.6 ± 11.4
1g	5	20.3 ± 34.6	0.7 ± 1.1	1.3 ± 1.1	1.5 ± 1.0	1.4 ± 1.1	2.1 ± 1.8	16.4 ± 24.1
1i	5	0.4 ± 0.1	6.5 ± 6.1	1.2 ± 0.6	1.7 ± 2.9	0.2 ± 0.2	4.2 ± 4.0	5.2 ± 3.8
1j	5	0.5 ± 0.8	3.6 ± 3.6	3.1 ± 1.7	2.9 ± 2.2	0.6 ± 0.6	0.5 ± 0.5	32.3 ± 33.1
1k	5	1.5 ± 2.5	1.2 ± 0.2	0.1 ± 0.1	2.8 ± 2.2	0.3 ± 0.3	1.4 ± 1.4	0.0 ± 0.0
1l	5	0.1 ± 0.2	7.6 ± 3.0	0.0 ± 0.0	0.4 ± 0.6	0.6 ± 0.7	0.0 ± 0.0	1.8 ± 3.2
3	5	0.1 ± 0.1	1.5 ± 2.1	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	1.3 ± 1.9	0.0 ± 0.0
Melphalan	5	0.8 ± 0.5	5.1 ± 6.2	21.4 ± 15.8	21.1 ± 5.1	0.8 ± 0.7	0.0 ± 0.0	0.4 ± 0.7
Curcumin	5	1.6 ± 1.1	12.8 ± 8.0	5.0 ± 2.6	21.3 ± 1.4	0.0 ± 0.0	0.6 ± 1.1	4.5 ± 7.4

Series **2** compounds were screened against human Hs27 fibroblast, Jurkat and Nalm-6 lymphocytic leukemia cells at a concentration of 1 μM. These data are presented in Table 4-4. The activity was expressed as the growth inhibition percentages.

Table 4-4: Evaluation of **2a-d** against Jurkat, Nalm-6, and Hs27 cell lines[60]

Compd.	% Growth Inhibition[a]				
	Jurkat	SI[b]	Nalm-6	SI[b]	Hs27
2a	19.8±26.3	49.5	28.0±7.0	70.0	0.4±0.7
2b	65.3±7.0	>65.3	65.1±24.1	>65.1	0.0±0.0
2c	13.2±15.7	>13.2	36.3±28.0	>36.3	0.0±0.0
2d	6.7±11.7	0.40	25.6±23.4	1.54	16.6±17.0

[a] The figures indicate the percentage inhibition of growth at a concentration of 1 μM for each compound.

[b] The letters SI indicate the selectivity index which is the ratio of the percentage inhibition of Jurkat or Nalm-6 lymphomas to the Hs27 cells.

Compound **2b** was extensively evaluated at concentration of 1 μM towards various human neoplasms namely murine EL4 T-lymphoma, human CEM T, RAJI B,

BJAB B, RAMOS B, SUPT-1 T-lymphoma cells, human HUT-102 T-, MOLT-3 T- cells leukemia, human HT-29 and COLO 205 colon cancer cells, human LAPC4 prostate cancer cells, and HCC70 breast cancer cells. The cytotoxic potencies of the compounds were expressed as the growth inhibition percentages, and indicated in Table 4-5.

Table 4-5: Evaluation of **2b** against twelve cancer cell lines at 1 μM[60]

Cell Line	% Growth Inhibition[a]
RAJI B	69.5 ± 10.6
BJAB B	13.2 ± 14.0
Ramos	89.3 ± 1.3
HUT-102	2.2 ± 2.9
Molt-3	15.3 ± 21.6
Sup-T1	44.5 ± 42. 6
EL-4	57.7 ± 16.3
HT-29	23.0 ± 20.4
COLO 205	11.7 ± 20.2
LAPC4	18.9 ± 19.3
HCC70	10.0 ± 14.3
CEM T	82.2 ± 18.3

[a] The figures indicate the percentage inhibition of growth at concentration of 1 μM for each compound.

Compounds in series **1** and **2** were also evaluated in another assay towards human HL-60 promyelocytic leukemic cells and human oral squamous cell carcinomas (HSC-2, HSC-3, and HSC-4). In addition, these compounds were evaluated against human HGF, HPC, HPLF non-malignant cells in order to examine the possible selectivity of the compounds towards neoplasms. The data expressed as CC_{50} values are presented in Table 4-6.

Table 4-6: Evaluation of **1a-m**, **2a-d**, melphalan, and curcumin against human tumor and normal cell lines[61]

Compound	Human tumor cell lines (CC_{50}) [a]								Human normal cell lines (CC_{50}) [a]			
	HSC-2	SI[b]	HSC-3	SI[b]	HSC-4	SI[b]	HL-60	SI[b]	HGF	HPC	HPLF	Ave[c]
1a	229±28	>1.70	>400	<0.97	308±61	>1.26	113±21	>3.44	>400	366±47	>400	>388.67
1b	380±12	>1.05	>400	<1.00	351±50	>1.14	150±31	>2.67	>400	>400	>400	>400.00
1c	143±8.4	2.03	268±21	1.08	191±16	1.52	42±16	6.91	324±3.0	202±8.7	345±3.5	290.33
1d	>400	<1.00	>400	<1.00	>400	<1.00	230±21	>1.74	>400	>400	>400	>400.00
1e	574±52	0.92	>800	<0.66	>800	<0.66	>800	<0.66	694±25	332±14	563±7	529.67
1f	167±20	1.33	283±8	0.78	19±5	11.68	554±28	0.40	286±3	139±18	241±6	222.00
1g	347±16	1.16	690±145	0.58	502±218	0.80	664±31	0.61	549±49	315±146	345±18	403.00
1h	75±2.0	2.96	161±19	1.38	139±7.0	1.60	57±11	3.90	285±11	127±5.3	255±21	222.33
1i	327±34	>1.70	342±9	>1.62	701±62	<0.79	>800	<0.69	>800	539±28	324±19	>554.33
1j	370±120	>2.16	495±31	>1.62	29±19	>27.59	390±21	>2.05	800>	800>	>800	>800.00
1k	410±26	0.98	287±20	1.40	334±18	1.20	705±16	0.57	519±13	253±22	432±54	401.33
1l	456±56	0.97	707±32	0.63	506±233	0.87	>800	<0.55	658±58	282±39	387±11	442.33
1m	54±6.4	3.98	153±6.0	1.41	134±17	1.60	66±30	3.26	270±11	142±2.5	233±28	215.00
2a	3.0±1.3	>95.67	51±25	>5.63	6.6±0.9	>43.48	23±4.2	>12.48	384±23	77±11	>400	>287.00
2b	31±4.6	4.98	50±5.5	3.09	63±6.0	2.45	32±11	4.82	123±25	240±17	100±17	154.33
2c	9.0±0.8	9.11	19±0.6	4.32	17±1.3	4.82	11±3.1	7.45	51±6.7	159±19	36±2.1	82.00
2d	9.8±3.8	>30.44	31±5.9	>9.62	26±3.2	>11.47	15±2.7	>19.89	>400	95±15	>400	>298.33
Melphalan	13.1±2.5	15.27	49±15	4.08	78±19	2.56	11±5.1	18.18	255±75	78±13	267±153	200.00
Curcumin	10±0.81	4.53	16±2.7	2.83	14±3.2	3.24	7.8±0.58	5.81	70±12	34±6.8	32±6.4	45.33

[a] The CC_{50} values are the concentrations of the compounds required to kill 50% of the cells.
[b] SI refers to the selectivity index, and represents the quotients of the average CC_{50} value of the compound towards normal cells and the CC_{50} figure generated for each neoplastic cell line.
[c] These figures are the average CC_{50} values of the compounds towards HGF, HPC and HPLF cell lines.

The target compounds **2a-d** were also screened in a MDR reversal assay at concentrations of 5 and 50 μg/mL in order to measure their MDR-revertant activity towards P-gp MDR cells. These data are presented in Table 4-7.

Table 4-7: Effect of **2a–d** on multidrug resistance in murine L5178 lymphoma cells[62]

Compd	**μg/ml**	**FAR[a]**
2a	5	1.57
	50	1.37
2b	5	3.53
	50	118.15
2c	5	1.91
	50	43.19
2d	5	3.41
	50	12.70
Verapamil	10	15.68

[a] The fluorescence activity ratio (FAR) values are the ratios of the fluorescence intensities of rhodamine 123 in treated and untreated murine L5178 lymphoma cells transfected with the human MDR-1 gene.

4.2 Discussion

The primary interest in this laboratory is the synthesis and bioevaluations of conjugated arylidene ketones designed as antineoplastic agents. These compounds have shown greater preferential affinity for thiols rather than amino and hydroxy groups.[65] Since thiols are absent in the nucleic acids, genotoxicity may be avoided by using these unsaturated ketones in comparison to contemporary anticancer drugs which generally interact with nucleic acids.[66] Initially, compounds which contain one 3-aryl-2-propenoyl group (ARCH=CHCO) were prepared. However, various studies revealed that the inclusion of a dienone moiety into candidate cytotoxins led to the capacity for a sequential attack on the olefinic carbon atoms which can be more detrimental to malignant cells than normal tissues. [67,68] Therefore, the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore (ARCH=CHCOCH=CHAR) has been included in the design of a number of antineoplastic agents.[69]

The rationale for preparing the auxiliary binders and the information obtained from these biodata in regards to producing directions for development of the project in the future is now presented. The auxiliary binders **1a-m** were designed to be incorporated into the target compounds. In order to examine the viability of the hypothesis postulated earlier, three esters **2b-d** were synthesized and evaluated for their cytotoxic potencies. The target compounds **2b-d** are esters and consequently most of the auxiliary binders are prepared as such. The biological evaluation of **1a-d** should point out whether the rates of hydrolysis or the differences in lipophilicity influence the cytotoxic potencies. In addition, the significance of the basic centre in **1a** should be addressed; hence, its

bioactivity could be compared with that of **1e-g**. The basic centre might not be required for the cytotoxic activity, and therefore the synthesis and bioevaluations of **1h** and **1m** was proposed. Another feature of these compounds is the spacer group between the aryl ring and the basic centre. Hence, comparing the IC_{50} values of **1i** and **1e** should enable an estimate of the contribution of the spacer group to cytotoxic potencies. Together with this consideration, the relative positions of the ethyl ester group and the diethylaminoethoxy and dimethylaminoethoxy are of importance. Thus, a small series of analogs **1j,k** and **1l** were prepared to compare their bioactivities with **1a** and **1e**.

All of the compounds in series **1** and **2** were evaluated against murine L1210 lymphocytic leukemia cells and human Molt 4/C8 and CEM T-lymphocytes as well as human cervical cancer HeLa cells. These biodata are presented in Tables 4-1 and 4-2. The L1210 screen was employed because of the sensitivity of these cells towards a number of anticancer drugs.[70] The Molt 4/C8, CEM, and HeLa assays were undertaken in order to examine whether the compounds have any effects on transformed and malignant human cells. These biological data may identify lead compounds. Comparisons between the IC_{50} values of various compounds were undertaken in order to seek any correlations between the structures of series **1** and **2** and their cytotoxic potencies. The standard deviations were taken into account in making these comparisons.

The IC_{50} values of series **1** against the four cell lines are presented in Table 4-1. Eight comparisons (I-VIII) between the IC_{50} values of various compounds were made in an effort to discern any structure-activity relationships (SAR). When comparing the

cytotoxic potencies of more than two compounds, the compound(s) with the lowest IC_{50} values are assigned as the potent compound(s). These comparisons are presented in Table 4-8.

Table 4-8: Comparisons between the cytotoxic potencies of some of the compounds in series **1a-m** and **3**

No.	Comparison	Cell Line			
		L1210	Molt 4/C8	CEM	HeLa
I	1a, 1e, 1f, 1g	1a, 1e, 1f > 1g	1f > 1a, 1e, 1g	1a, 1e, 1f > 1g	1a, 1f, 1g > 1e
II	1a, 3	1a > 3	1a = 3	1a > 3	1a > 3
III	1a-d	1c > 1a, 1b, 1d	1c > 1a, 1b, 1d	1a = 1b, 1c, 1d	1a = 1b, 1c, 1d
IV	1e, 1i	1i >1e	1i >1e	1i >1e	1i >1e
V	1a, 1j, 1k	1a, 1k > 1j	1k > 1a, 1j	1a = 1k, 1j	1a = 1k, 1j
VI	1k, 1l	1k = 1l	1k > 1l	1k > 1l	1k = 1l
VII	1e, 1h	1h > 1e	1h > 1e	1h > 1e	1h > 1e
VIII	1h, 1m	1h > 1m	1h > 1m	1h > 1m	1h = 1m

(I) Comparing the amino group in the 4-alkylaminoethoxy substituents revealed that the relative potencies are **1f** > **1a** > **1e** > **1g**. This result may be due to the lipophilicity of **1f** and **1a** which have clogP values of approximately 4 whereas in the case of **1e** and **1d**, the clogP figures are approximately 3. (II) The reduction of the polarity of the carboxylic acid group in **3** by its conversion into the corresponding ethyl ester **1a** led to increases in the cytotoxic potency generally and this result could be due to

increases in both lipophilicity and membrane permeability. (III) Comparing the cytotoxic potencies of the potential prodrugs of **3** namely **1a-d** revealed that the isopropyl analog **1c** is more cytotoxic than the other esters **1a**, **1b**, **1d**. The isopropyl ester has the highest lipophilicity which may contribute to the increased cytotoxicity. (IV) The ester **1i**, which has the trimethylene spacer between the nitrogen and oxygen atoms, is more potent than the dimethylene spacer analog **1e**. Therefore, the distance between the nitrogen and oxygen atoms in the alkylaminoalkoxy substituents, which are able to form hydrogen bonds at the receptor, should be investigated in order to obtain more potent auxiliary binders. (V) In **1j**, **1k**, and **1a**, the 2-diethylaminoethoxy substituents are placed in the ortho-, meta-, and para- positions of the aryl ring, respectively. The order of potency is **1k** > **1a** > **1j** which indicates that the ortho structural isomer is the least potent, and the meta- structural isomer has the maximum potency. (VI) Comparing the dialkylamino group in the compounds containing meta- 2-alkylaminoethoxy substituents **1k** and **1l** revealed that the diethylamino analog **1k** is more potent than the dimethylamino analog **1l**. This result may be due to the higher lipophilicity of **1k** which has a clogP value of approximately 4. (VII) The isosteric replacement of the basic centre in **1e** by a methine group led to **1h** which has increased cytotoxic potency compared to **1e**. In fact, **1h** displayed the most potent cytotoxicity in series **1** towards both T-lymphocytes. That could be due to its having the highest clogP value among the compounds in series **1**. (VIII) Comparing the ethyl **1h** and methyl **1m** esters revealed that the ethyl ester is more potent which might be due to the reduced clogP value in **1m**.

In conclusion, these biodata could be utilized in designing modified molecules in the future in order to obtain more cytotoxic molecules, e.g., by considering the optimal aryl substituents, as well as the optimal ester groups. Specifically **1h** is the most potent molecule having an average IC_{50} value of 61 μM towards both T-lymphocytes. Moreover, the lipophilicity of the molecules based on the clogP values appears to display a significant role in the relative potencies of the compounds.

The target compounds **2b-d** were prepared as hybrids (Figure 4-2) between a candidate cytotoxin **2a** and the auxiliary binders **1a**, **f**, and **1g**. The cytotoxicity of **2a** in these bioassays has not been reported previously and therefore its potency needed to be determined. The bioevaluations of **2b-d** was planned in order that the following comparisons of potencies between different compounds could be undertaken. First, the IC_{50} values of **2b-d** can be compared with the analog which has no substituents in the arylidene aryl rings. In other words, does the incorporation of the auxiliary binder group onto these aryl rings decrease the IC_{50} values? Second, since **2b-d** are esters, the possibility exist that these compounds will hydrolyze to **2a** and various aromatic acids. Hence, comparisons between the potencies of **2b-d** with **2a** was planned. Third, the amino groups in **2b-d** have different hydrophobic and electronic properties which may be reflected in changes in their cytotoxic potencies.

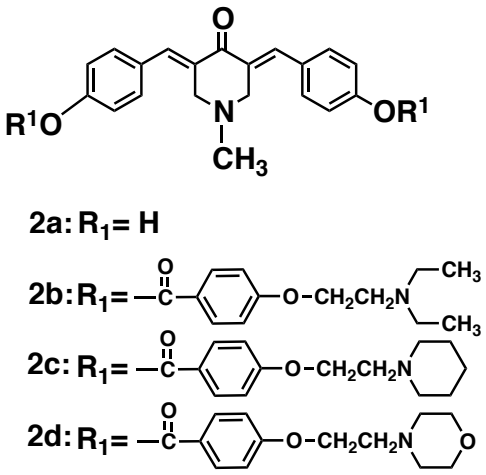


Figure 4-2: General structures of the compounds **2a-d**

Comparisons were also made between the IC_{50} values of the target compounds **2b-d** and series **4** in an effort to discern the effect of the auxiliary binders on cytotoxic potencies. Both series **2** and **4** share a structural similarity with the non-enolic form of curcumin which possesses two 3-aryl-2-propenoyl groups (ARCH=CHCO-). Hence, the cytotoxic potencies of these compounds were compared to curcumin. In addition, the potencies of the compounds were compared to melphalan which is an alkylating agent used in cancer chemotherapy. The IC_{50} values of series **2** against four cell lines are presented in Table 4-2.

Compound **2a** has IC_{50} values below 20 μM against both T-lymphocytes as well as the HeLa bioassay, and is equipotent with curcumin towards CEM and HeLa cells. The biodata for compounds **2b-d** revealed that IC_{50} values were below 20 μM for **2b** in the L1210 assay, **2c** in the L1210, CEM, and HeLa screens, and **2d** in the L1210, Molt 4/C8, and HeLa assays. Comparing the three analogs **2b-d**, the IC_{50} figures reveal that **2c** and

2d are generally more potent than **2b**. The three analogs **2b-d** also displayed greater cytotoxic potencies than curcumin in some assays namely **2b** in the L1210 assay, **2c** in L1210 and the HeLa screens, and **2d** in the L1210 assay. Equipotency with curcumin was observed for **2b** in the HeLa screens, **2c** in the CEM and HeLa screens, and **2d** in HeLa assay. Moreover, comparing the IC_{50} values of **2a-d** with melphalan revealed that **2c** and **2d** were equipotent with this reference compound against L1210 cells.

The investigation was undertaken to examine whether the attachment of the 4-(2-aminoethoxy)phenylcarbonyloxy group to the aryl rings of **4a**, as well as, the substitution of methyl group in **4b** by the 4-(2-aminoethoxy)phenylcarbonyloxy substituent and the substitution of methyl group in **4c** by the 4-(2-aminoethoxy)phenylcarbonyl substituent will increase or decrease the cytotoxic potency. The biodata are presented in Table 4-2, and reveal that in relative to **4a**, the incorporating of the auxiliary binder in **2c** increased the cytotoxic potencies in the L1210 assay, but not in T-lymphocytes assays. In relation to **4b**, the incorporation of the 4-(2-aminoethoxy)phenylcarbonyloxy group led to a significant increase the potent cytotoxicity in all the three assays in general. In comparison with **4c**, the incorporating of the auxiliary binders **2b**, **2c**, and **2d** increased the cytotoxic potencies in the L1210 and the T-lymphocytes assays (Table 4-9).

Table 4-9: Comparison of the cytotoxic potencies of **4a-c** and the hybrid molecules **2b-d**

Compd	IC_{50} Ratio [a]								
	L1210			Molt 4/C8			CEM		
	4a	4b	4c	4a	4b	4c	4a	4b	4c
2b	0.84	29.1	26.8	0.05	6.4	5.28	0.12	8.4	6.19
2c	1.45	50.4	46.5	0.09	12.4	10.3	0.20	14.12	10.42
2d	1.12	39.0	35.9	0.17	23.1	19.2	0.11	7.57	5.6

[a] The figures of the ratios of the IC_{50} values of **4a**, **4b**, and **4c** divided by the IC_{50} values for **2b-d**.

A further biological evaluation of the representative auxiliary binders and the target compounds was undertaken in order to assess the extent of their cytotoxic potencies. Non-adherent malignant cells are capable of invading other organs after metastasis has occurred. Compounds with growth-inhibitory properties towards non-adherent cells may therefore have the ability to prevent metastasis. The non-adherent cells are Jurkat, BJAB, Nalm-6, EL-4, CEM, Sup-T1, Ramos, and BW5147 cancer cells, Hs27 non-malignant cells, while the adherent cancer cells are LNcAP, DU145, RAJI B, HUT-102, Molt-3, HT-29,COLO 205, LAPC4, and HCC70. The auxiliary binders **1e-g**, **i-l** and **3** were screened against non-adherent and adherent malignant cells namely human LNcAP, DU145 prostate malignant cells, human Jurkat and BW5147 T-cell lymphomas, murine EL-4 T-cell lymphoma, human BJAB and Nalm-6 B-cell lymphomas (Table 4-3). Series **2** were evaluated also against malignant non-adherent cells namely Jurkat T-

lymphoma and Nalm-6 pre-B-lymphoma. Moreover, the non-malignant human Hs-27 fibroblast cell line was employed to examine whether the compounds have greater potency towards neoplasms than non-malignant cells. One candidate **2b** was broadly screened in twelve assays including lymphoma, leukemia, colon, prostate, and breast cancer cells. These cells are human RAJI, BJAB, and Ramos B-lymphomas, human Sup-T1, CEM, and murine EL-4 T-lymphomas, human HUT-102 and Molt-3 T-cell leukemia cells, HT-29 and COLO 205 colon cancer cells, LAPC4 prostate cancer cells, and HCC70 breast cancer cells. These biological data are presented in Tables 4-3, 4-4, and 4-5, respectively.

A comparison between the percentages of growth inhibition of various compounds was undertaken at a concentration of 5 μM in order to seek any correlations between the structures of series **1** and their cytotoxic potencies. The standard deviations were taken into account in making these comparisons. However, it can be concluded that the percentages of growth inhibition of the compounds were below 10% in all assays, except for **1g** towards Jurkat and DU145 cells and **1j** against DU145 cells.

The growth inhibition of compounds **2a-d** against the three cell lines Jurkat, Nalm-6, and Hs-27 are presented in Table 4-4. A comparison between the percentages of growth inhibition of various compounds was made in an effort to discern any structure activity relationships (SAR). These biological data revealed that **2b** is the most potent compound in series **2** and **2b** is a lead compound. Compound **2b** was the most potent in Jurkat assay, having a growth inhibition more than 50%. On the other hand, **2a**, **2c**, and

2d were equipotent towards Jurkat cells. In addition, **2b** inhibited the growth of Nalm-6 cells in excess of 50%. The biodata also reveals that both **2a-c** are selective towards the malignant cells. The analog **2a**, as well as the hybrid molecule **2d** displayed toxicity towards non-malignant cells Hs-27 as they inhibited these cells growth by 0.4% and 16.6% respectively.

Clearly, **2b** is a lead compound; hence, additional screening was undertaken using twelve cancer cell lines at the concentration of 1 μM in order to examine its sensitivity towards various non-adherent lymphoma, leukemia, prostate, breast, and colon cancers (Table 4-5). The candidate **2b** exhibited greatest potency towards RAMOS, RAJI, and EL4 cancer cell lines in which the percentages of growth inhibition are approximately above 90%, 70%, and 60% respectively. Hence, the IC_{50} values of **2b** towards these three cell lines are in the sub-micromolar range, indicating that **2b** is a lead molecule for further development.

A further cytotoxicity study was undertaken in order to investigate whether the compounds are not only cytotoxic to neoplasms but have greater lethality to malignant cell lines. All of the compounds in series **1** and **2** were evaluated against four human neoplastic cell lines namely HL-60 promyelocytic leukemic cells as well as HSC-2, HSC-3 and HSC-4 oral squamous cell carcinomas. In addition, they were screened against three human normal cells namely HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. These biodata are presented in Table 4-6.

The biodata of series **1** presented in Table 4-6 indicate that most of the compounds displayed cytotoxicity towards neoplasms at CC_{50} values higher than 100 μM. However, some of the series **1** compounds displayed cytotoxic potency towards leukemia and specific squamous cells at CC_{50} values between 19-75 μM. The potencies in μM are given as follow: **1c** 42 (HL-60), **1f** 19 (HSC-4), **1h** 57 and 75 (HL-60 and HSC-2 respectively), **1j** 29 (HSC-4), and **1m** 54 and 66 (HSC-2 and HL-60) respectively.

The biodata presented also reveal that **2a-d** CC_{50} average values against the four cell lines are 20.9, 44.0, 14.0, and 20.5 μM respectively. The order of potencies are therefore **2c** > **2a,d** > **2b**. Comparison of the CC_{50} values of **2a-d** and melphalan in the three squamous cells carcinomas as well as leukemic cells reveal that **2a** (3 and 6.6 μM), **2c** (9 and 17 μM), and **2d** (9.8 and 26 μM) in HSC-2 and HSC-4 assays are more potent than melphalan. In addition, compound **2c** displayed more potency (19 μM) than melphalan in the HSC-3 assay, whereas **2b** is equipotent in the HSC-4 assay (63 μM). Significantly, 11 fold more potency than melphalan in the HSC-2 and HSC-4 assays (63 μM) respectively, while **2c** possess 1.5 and 4.6 folds more potency in the same assays, respectively.

In order to address the compounds' selective toxicity to neoplasms than normal cells, selectivity index (SI) figures were calculated for **1a-m** and **2a-d**. Under clinical conditions, the tumor cells are surrounded by a number of different types of normal cells and in order to stimulate the in vivo situations, the average CC_{50} value of the compounds towards the three normal cell lines was divided by the CC_{50} figure generated using each

neoplastic cell line. These data are presented in Table 4-6. 54% of the auxiliary binders **1** have SI figures of greater than 1, while 46% have SI figures of lower than 1. All of the compounds in series **2** have SI average of greater than 1 which indicates that their toxicity towards normal cells are lower than to the neoplasms. These average figures are **2a** (36.8), **2b** (2.18), **2c** (2.69), and **2d** (16.34), and it indicates that all of series **2** compounds are more selective than the reference compounds melphalan and curcumin; in addition, **2a** and **2d** displayed greater selectivity than **2b** and **2c**.

Multidrug resistance development is one of the main problems in cancer chemotherapy. Compounds that revert MDR may also exhibit antineoplastic activity, or if not, they can be co-administered with anticancer drug(s). MDR-revertants in both cases help in reducing the dose given and consequently potential toxicity is reduced. In order to determine whether a compound possesses MDR-reversal activity, murine L-5178 lymphoma cells are transfected with the human MDR-1 gene.[71] Measuring the fluorescence intensities in the rhodamine 123 treated and untreated transfected and parental cells enables the calculation of the fluorescence activity ratio (FAR) values. The FAR ratios indicate the magnitude of the MDR-reversal properties of the compounds; if they are above the value of 1 this means the molecule possess reversal activity. All of the compounds were assessed using concentrations of 5 and 50 μg/mL and the data are presented in Table 4-7. It was reported previously from this laboratory that 3,5-bis-benzylidene-4-piperidones lack the MDR properties, while the N-acyl analogs **5** have high FAR values at a concentration of 4 μg/mL.[40] Series **5**, which has a 4-(2-aminoethoxy)phenylcarbonyl group attached to the piperidyl nitrogen atom, exhibited

substantial MDR-revertant activities. Hence, the placement of the 4-(2-aminoethoxy)phenylcarbonyloxy group to the arylidene aryl ring of **4a** may produce MDR-revertant activity. Series **2a-d** were screened in this assay using concentrations 5 and 50 μg/mL and these biodata are presented in Table 4-7. In the higher concentration, the FAR values are 1.37, 118, 43.2, and 12.7, respectively. It is clear that the hybrid molecules possess greater MDR-revertant activity than the hydroxy analog **2a**. Significantly, **2b** is the most potent compound and thus is considered a lead molecule. Although the potency of **2c** is 2.7 fold lower than that of the lead molecule, its activity is still encouraging. At the low concentration of 5 μg/mL, the FAR values of **2a**, **2b**, **2c**, and **2d** are 1.57, 3.53, 1.91, and 3.41 respectively. In comparison to the highest potencies of both **2a-d** at the high concentration of 50 μg/mL and to series **5** at 4 μg/mL, they are in general, less potent MDR-revertants. Due to the solubility issues, verapamil was used as a positive control at concentration of 10 μg/mL and its FAR value is 15.68.

CHAPTER 5

5. Conclusions

The aim of this project was the development of novel curcumin analogs containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore through the incorporation of auxiliary binders. These compounds are multi-targeted compounds, that is they could bind at the auxiliary binding site in addition to the interaction of the 1,5-diaryl-3-oxo-1,4-pentadienyl group at the primary binding site. Cytotoxicity activity is taking place inter alia through thiol alkylation, which results in depleting GSH levels in tumor cells. Auxiliary binders might strengthen binding to the receptor only, or they might exhibit cytotoxic potency as well. If auxiliary binders display cytotoxic effects, they would be acting at sites far from the primary binding site. Three target compounds **2b-d** and the auxiliary binders **1a-m** were synthesized using, on most occasions, various condensation reactions (pages 61-63), followed by their biological evaluation *in vitro* in order to examine the cytotoxic activity[59-61] as well as the MDR[62] properties of the candidate cytotoxins. In general some of the auxiliary binders and target compounds displayed encouraging cytotoxic potencies towards various human and animal malignant cell lines. As well, remarkable results were obtained in the MDR-reversal assay. The biological evaluation of the auxiliary binders **1a**, **1f**, and **1g** towards four cancer cells L1210, Molt4/C8, CEM, and HeLa revealed that they lack significant cytotoxic potency (IC_{50} > 100 μM). For the other auxiliary binders in series **1**, very low potencies were displayed (IC_{50} > 100 μM), except for one compound **1h** towards both T-lymphocytes (IC_{50}=61 μM). The attachment of **1a**, **1f**, and **1g** to **2a** led to the significant increased

potency of **2b-d** in the same assay (IC_{50} = 13.3-25.5 μM). These data are presented in Tables 4-1 and 4-2, pages 65,66.

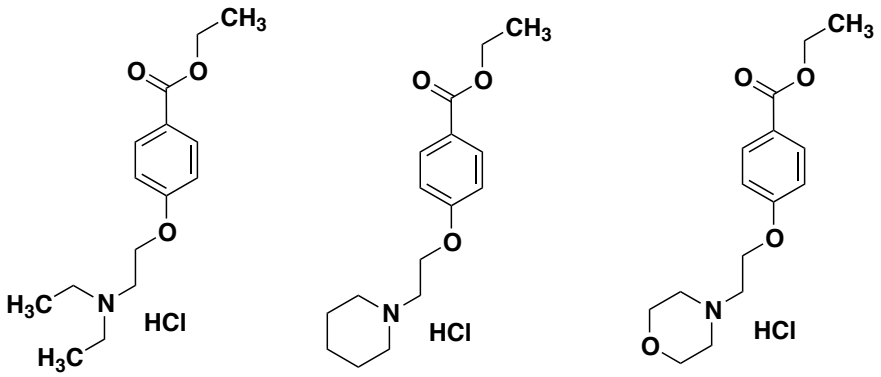


Figure I: Chemical structures of **1a**, **1f**, and **1g**

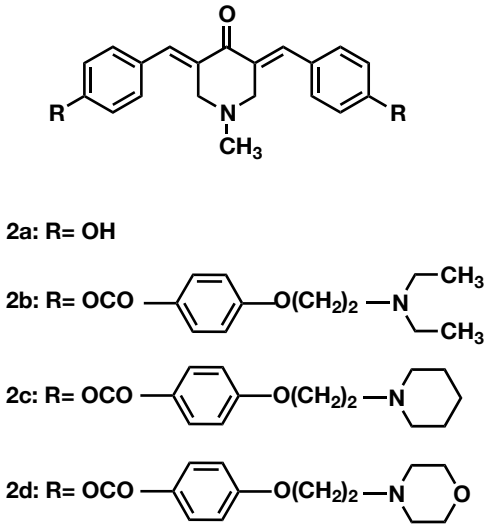


Figure II: Chemical structures of **2a-d**

To investigate the anti-metastasis activity of the target compounds **2b-d** as well as the auxiliary binders **1**, various adherent and non-adherent cancer cells (page 81) were

used and the percentage of growth inhibition was obtained[60]. The target compounds **2b-d** showed favorable cytotoxic potencies in which the inhibition of tumor growth took place at micromolar and sub-micromolar concentrations. The incorporation of the auxiliary binders **1a**, **1f**, and **1g** into **2b-d** led to potency increases revealing compounds with encouraging cytotoxic properties in this assay. The candidate **2b** displayed the maximum potency, that is, **2b** inhibited up to 70% of various adherent and non-adherent tumor cells at 1 μM (Table 4-5, page 70). As well, the selectivity towards malignant cells in contrast to normal cells was demonstrated mostly by the analogs **2b** (SI >65.5) and **2d** (SI >36.3) as indicated in Table 4-4. Among other auxiliary binders, two compounds **1g**, and **1j** displayed inhibitory effects at 5 μM toward Jurkat and DU145 cancer cells (Table 4-3, page 68). To investigate the selective cytotoxicity of target compounds, the compounds were screened towards various normal and cancer cells and the selectivity indicies were calculated[61] (Table 4-6, page 72). The target compounds **2b-d** as well as **2a** displayed favorable cytotoxicity at low CC_{50} values. The most potent compounds are **2c** (CC_{50}= 14 μM) and **2d** (CC_{50}= 21 μM). However, in terms of selectivity, compound **2d** is the lead compound (SI > 30.44). Compounds **2b-d** as well as **2a** display remarkable MDR reverting properties[62] at 50 μM, especially **2b** which displayed the most favorable FAR value at 50 μM. At a lower concentration (5 μM), the compounds' revertant properties were also revealed but with lower FAR values (Table 4-7, page 73). Future structural modifications should first include the other auxiliary binders in designing novel anticancer agents having the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore to improve the overall potency. Second, the mechanism of action of the auxiliary binding should also be investigated.

CHAPTER 6

References

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